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# CONTENTS OF VOLUME 122

No. 1, DECEMBER, 1937

	PAGE
GRAHAM, W. R., JR. The utilization of lactic acid by the lactating mammary gland . . . . .	1
SULLIVAN, M. X., and HESS, W. C. The effect of pyruvic acid on the estimation of cystine and cysteine . . . . .	11
HARTZLER, EVA R. A note on the determination of potassium by the method of Shohl and Bennett . . . . .	19
SNYDER, J. C. A note on the use of the Haldane apparatus for the analysis of gases containing ether vapor . . . . .	21
TOENNIES, GERRIT. Oxidation of cysteine in non-aqueous media. The "sulfenic acid" as the primary oxidation product . . . . .	27
KERR, STANLEY E., and ANTAKI, ALBERT. The carbohydrate metabolism of brain. V. The effect of certain narcotics and convulsant drugs upon the carbohydrate and phosphocreatine content of rabbit brain . . . . .	49
KERR, STANLEY E. Note on the phosphorus content of rat brain in experimental rickets . . . . .	53
STEKOL, JAKOB A. Studies on the mercapturic acid synthesis in animals. VII. Bromobenzene and <i>l</i> -cystine in relation to growth of rats on a navy bean meal diet . . . . .	55
MILLER, MAX. The diffusible calcium of serum and transudates <i>in vivo</i> . . . . .	59
MILLER, MAX. Ionized calcium of serum and transudates <i>in vivo</i> . . . . .	71
MAN, EVELYN B., and GILDEA, EDWIN F. Notes on the extraction and saponification of lipids from blood and blood serum . . . . .	77
JOHNSON, MARVIN J. Specificity of intestinal aminopolypeptidase . . . . .	89
EMERSON, OLIVER H., EMERSON, GLADYS A., MOHAMMAD, ALI, and EVANS, HERBERT M. The chemistry of vitamin E. Tocopherols from various sources . . . . .	99
SMITH, PAUL K., TAYLOR, ALICE C., and SMITH, ELIZABETH R. B. Thermodynamic properties of solutions of amino acids and related substances. III. The ionization of aliphatic amino acids in aqueous solution from one to fifty degrees . . . . .	109
BALLS, A. K., MATLACK, M. B., and TUCKER, I. W. The hydrolysis of glycerides by crude pancreas lipase . . . . .	125
DOTY, J. R., and EATON, A. G. Metabolism of the basic amino acids. I. Rates of absorption in rats of the monohydrochlorides of <i>d</i> -lysine and <i>l</i> -histidine . . . . .	139
BOYD, ELDON M., and STEVENSON, J. W. The regeneration of blood lipids following a single massive hemorrhage in rabbits . . . . .	147

CHARGAFF, ERWIN, and OLSON, KENNETH B. Studies on the chemistry of blood coagulation. VI. Studies on the action of heparin and other anticoagulants. The influence of protamine on the anticoagulant effect <i>in vivo</i> .....	153
PERLMAN, I., RUBEN, S., and CHAIKOFF, I. L. Radioactive phosphorus as an indicator of phospholipid metabolism. I. The rate of formation and destruction of phospholipids in the fasting rat..	169
SCHULTZ, HAROLD W., and MATTILL, H. A. Studies on the vitamin B complex.....	183
LEVENE, P. A., TIPSON, R. STUART, and KREIDER, LEONARD C. Conversion of uronic acids into corresponding hexoses. II. Catalytic reduction of the methyl ester of 2,3,4-trimethyl $\alpha$ -methyl-d-galacturonide.....	199
LEVENE, P. A., and CHRISTMAN, CLARENCE C. Conversion of uronic acids into corresponding hexoses. III. Catalytic reduction and deacetylation of the methyl ester of 2,3,4-triacetyl $\alpha$ -methyl-d-galacturonide.....	203
WOOLLEY, D. W., and PETERSON, W. H. Some observations on the Kapeller-Adler method for the determination of histidine. The histidine content of yeast.....	207
WOOLLEY, D. W., and PETERSON, W. H. The chemistry of mold tissue. XIV. Isolation of cyclic choline sulfate from <i>Aspergillus sydowi</i> ...	213
WHITE, ABRAHAM, and BEACH, ELIOT F. The rôle of cystine, methionine, and homocystine in the nutrition of the rat.....	219
KESTON, ALBERT S., RITTENBERG, D., and SCHOENHEIMER, RUDOLF. Determination of deuterium in organic compounds.....	227
WYCKOFF, RALPH W. G. An ultracentrifugal study of the pH stability of tobacco mosaic virus protein. Plates 1 and 2.....	239
HEYMANN, WALTER. Metabolism and mode of action of vitamin D. IV. Importance of bile in the absorption and excretion of vitamin D.....	249
HEYMANN, WALTER. Metabolism and mode of action of vitamin D. V. Intestinal excretion of vitamin D.....	257
MARSHALL, E. K., JR. Determination of sulfanilamide in blood and urine.....	263

## NO. 2, JANUARY, 1938

GRAHAM, W. R., JR., PETERSON, V. E., HOUCHIN, O. B., and TURNER, C. W. The utilization of fractions of the nitrogen partition of the blood by the active mammary gland.....	275
BOYDEN, RUTH, and POTTER, V. R. On the form of copper in blood plasma.....	285
LEVENE, P. A., and KUNA, MARTIN. The configurational relationship of 3-aminoheptane to that of norleucine.....	291
EHRENSTEIN, MAXIMILIAN, and COREY, EDWARD L. Activation of testosterone by higher fatty acids and their acid sodium salts....	297

HIRSCHMANN, H., and WINTERSTEINER, OSKAR. The isolation of estrogenic diols from the urine of pregnant mares. ....	303
EICHELBERGER, LILLIAN. The exchange of salt and water between muscle and blood. IV. Correction of values for volume phases of skeletal muscle. Methods for determination of blood volume in muscle. ....	323
STEKOL, JAKOB A. Studies on the mercapturic acid synthesis in animals. VIII. <i>l</i> -Cystine, <i>dl</i> -methionine, glutathione, and taurine in relation to the synthesis of mercapturic acids in the rat. ....	333
CARPENTER, THORNE M. The partition of urinary nitrogen of fasting and hibernating woodchucks ( <i>Arctomys monax</i> ). ....	343
DU VIGNEAUD, VINCENT, and IRISH, OLIVER J. The rôle of the acetyl derivative as an intermediary stage in the biological synthesis of amino acids from keto acids. ....	349
STERN, KURT G., and WHITE, ABRAHAM. Studies on the constitution of insulin. III. The acetylation of insulin by ketene. ....	371
SCHANTZ, E. J., ELVEHJEM, C. A., and HART, E. B. The relation of fat to the utilization of lactose in milk. ....	381
EVELYN, KENNETH A., and GIBSON, JOHN G., 2ND. A new type of absorption cell for the photoelectric microcolorimeter. ....	391
SCHULTZE, M. O., STOTZ, ELMER, and KING, C. G. Studies on the reduction of dehydroascorbic acid by guinea pig tissues. ....	395
STOTZ, ELMER, HARRER, CARTER J., SCHULTZE, M. O., and KING, C. G. The oxidation of ascorbic acid in the presence of guinea pig liver. ....	407
JACOBS, WALTER A., and CRAIG, LYMAN C. The ergot alkaloids. XIII. The precursors of pyruvic and isobutyrylformic acids. ....	419
JACKSON, RICHARD W., and BLOCK, RICHARD J. The metabolism of cystine and methionine. II. The availability of <i>d</i> - and <i>l</i> -methionine and their formyl derivatives in the promotion of growth. ....	425
KOCHAKIAN, CHARLES D., MACLACHLAN, P. L., and McEWEN, H. DOUGLAS. Do androgens affect the blood lipids? ....	433
MORISON, ROBERT S., McLEAN, REGINA, and JACKSON, ELIZABETH B. Observations on the relation between ionized and total calcium in normal and abnormal sera and their ultrafiltrates. ....	439
WALD, GEORGE, and ZUSSMAN, HYMAN. Carotenoids of the chicken retina. ....	449
STERN, KURT G., and SALOMON, KURT. On ovoverdin, the carotenoid-protein pigment of the egg of the lobster. ....	461
KURTZ, ALTON C. A simple synthesis of <i>dl</i> -citrulline. ....	477
IOB, VIVIAN, and SWANSON, W. W. The extracellular and intracellular water in bone and cartilage. ....	485
SNYDER, FRED H., and CORLEY, RALPH C. Amino acid catabolism. V. The influence of structural configuration on the deamination of $\alpha$ -amino acids in the normal dog. ....	491
HILDITCH, T. P., and LONGENECKER, H. E. Further determination and characterization of the component acids of butter fat. ....	497

BERNSTEIN, SAMUEL S., JONES, ROBERT L., ERICKSON, BETTY NIMS, WILLIAMS, HAROLD H., AVRIN, IRA, and MACY, ICIE G. A method for the preparation of posthemolytic residue or stroma of erythrocytes.....	507
ERICKSON, BETTY NIMS, WILLIAMS, HAROLD H., BERNSTEIN, SAMUEL S., AVRIN, IRA, JONES, ROBERT L., and MACY, ICIE G. The lipid distribution of posthemolytic residue or stroma of erythrocytes....	515
GOERNER, ALFRED. Effect of dibenzanthracene on vitamin A and total lipid of mitochondria.....	529
SCUDI, JOHN V. The determination of sulfanilamide ( <i>p</i> -aminobenzene-sulfonamide) in biological media.....	539
LINWEAVER, HANS. The solubility and chemical and physical absorption of nitrogen gas in <i>Azotobacter</i> cells.....	549
BERGMANN, MAX. Complex salts of amino acids and peptides. III. Salts of dioxalatodipyridinochromiato acid (dioxpyridic acid) and dioxalatodianilinochromiato acid (dioxanilic acid) .....	569
BERGMANN, MAX, and NIEMANN, CARL. On the structure of silk fibroin.....	577

## NO. 3, FEBRUARY, 1938

MALLOY, HELGA TAIT, and EVELYN, KENNETH A. Oxidation method for bilirubin determinations in bile and meconium with the photo-electric colorimeter.....	597
WEST, HAROLD D., KRUMMEL, GUY S., and CARTER, HERBERT E. Synthesis of $\alpha$ -amino- $\beta$ -hydroxy- <i>n</i> -butyric acids. V. Preparation of <i>dl</i> -allothreonine, with notes concerning the addition of methyl hypobromite to unsaturated acids.....	605
WEST, HAROLD D., and CARTER, HERBERT E. Synthesis of $\alpha$ -amino- $\beta$ -hydroxy- <i>n</i> -butyric acids. VI. Preparation of <i>d</i> - and <i>l</i> -allothreonine and nutritive value of the four isomers.....	611
LORENZ, F. W., ENTENMAN, C., and CHAIKOFF, I. L. The influence of age, sex, and ovarian activity on the blood lipids of the domestic fowl.....	619
CAMPBELL, HAROLD A., and LINK, KARL PAUL. Acetals in the sugar group. I. The dimethyl acetal of <i>d</i> -galactose.....	635
MOYER, LAURENCE S. Electrokinetic aspects of surface chemistry. III. A comparison of the microscopic and moving boundary methods of electrophoresis.....	641
GOEBEL, WALTHER F. Derivatives of glucuronic acid. VIII. The structure of benzoylglucuronic acid.....	649
BROWN, W. L. The influence of pimiento pigments on the color of the egg yolk of fowls.....	655
LEVENE, P. A., and CHRISTMAN, CLARENCE C. Conversion of uronic acids into corresponding hexoses. IV. Catalytic reduction of the methyl ester of diacetone <i>d</i> -galacturonic acid.....	661
SOBEL, ALBERT E., and SKLERSKY, SAMUEL. A direct acidimetric microtitration method for calcium.....	665

MINDLIN, ROWLAND L., and BUTLER, ALLAN M. The determination of ascorbic acid in plasma; a macromethod and micromethod . . .	673
ANDREWS, JAMES C. Reduction of certain sulfur compounds to hydrogen sulfide by the intestinal microorganisms of the dog . . . . .	687
TUFTS, ELMA V., and GREENBERG, DAVID M. The biochemistry of magnesium deficiency. I. Chemical changes resulting from magnesium deprivation . . . . .	693
TUFTS, ELMA V., and GREENBERG, DAVID M. The biochemistry of magnesium deficiency. II. The minimum magnesium requirement for growth, gestation, and lactation, and the effect of the dietary calcium level thereon . . . . .	715
RICHARDS, MARIANNA M. The effect of glycine upon the activity coefficient of glycine, egg albumin, and carboxyhemoglobin . . . . .	727
JONES, D. BREESE, GERSDORFF, CHARLES E. F., and PHILLIPS, SAMMIE. Proteins of the black bean of the Mayas, <i>Phaseolus vulgaris</i> . . . . .	745
SCHMIDT, E. G. The determination of sulfanilamide in tungstic acid blood filtrates by means of sodium $\beta$ -naphthoquinone-4-sulfonate . .	757
KOEPFLI, J. B., THIMANN, KENNETH V., and WENT, F. W. Phytohormones: structure and physiological activity. I . . . . .	763
BAERNSTEIN, HARRY D. The nutritional value of various protein fractions of the peanut . . . . .	781
INDEX TO VOLUME 122 . . . . .	791



# THE UTILIZATION OF LACTIC ACID BY THE LACTATING MAMMARY GLAND\*

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The experiments of Kaufman and Magne (1), Turner and Herman (2), Blackwood and Stirling (3), Lintzel (4), and Graham, Jones, and Kay (5) show that considerable quantities of sugar are removed from the blood as it passes through the mammary gland.

Foa (6) was able to obtain secretion of a milky substance from the perfused surviving mammary gland only when the perfusion fluid contained glucosc. Nitzescu and Nicoleau (7), Bucciard (8), Macchiarulo (9), Gowen and Tobey (10), and Brown *et al.* (11) have shown that a decline in the lactose percentage and in the total yield of milk accompanies a diminished sugar level in the blood of lactating animals after insulin or inanition experiments. Interference with the carbohydrate metabolism by the use of phlorhizin causes similar declines in milk yield (Patton and Cathcart (12); Gowen and Tobey ((10) p. 45). Grant (13), using a tissue culture technique, has shown that glucose was the only hexose of a number used which could be rapidly converted into lactose by the mammary gland slice under these conditions.

The evidence cited, supported by many other experiments, has led to the belief that glucose is the precursor of lactose and that glucose alone supports the carbohydrate metabolism of the mammary gland. With this fact apparently substantially established

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and with newly developed techniques for obtaining arterial blood samples and measurements of blood volume flow, the time seemed appropriate for an attempt to study the balance of carbohydrate metabolism in the mammary gland during lactation. This paper reports such experiments and shows that, in addition to glucose, lactic acid may serve as a precursor of lactose.

#### EXPERIMENTAL

Lactating goats were surgically prepared for the experiments by (a) exteriorization of the carotid artery, (b) removal of one mammary gland, and (c) tying and cutting the external pudic and perineal veins, thus leaving one major inlet and one major outlet for the blood flowing through the gland. Arterial and mammary venous blood samples were taken simultaneously with

TABLE I

*Results of Attempted Balance Experiments between Removal of Glucose from Blood and Production of Lactose by Mammary Gland*

Time of experiment	Blood volume flow	Mean blood sugar difference (arterial minus venous)	Absolute sugar removed	Lactose secreted
<i>min.</i>	<i>ml per min</i>	<i>mg per cent</i>	<i>gm</i>	<i>gm</i>
340	161	9 42	5 15	12 62
170	145	12 8	3 16	6 06

strict attention to the observations previously made in taking such samples for the purpose of comparisons in the light of milk secretion (Graham *et al.* (14)). Blood flow was measured by the Herrick and Baldes (15) modification of the thermostromuhr technique. No anesthetics were used in the experimental periods, the animal having been previously conditioned to lying quietly on the table designed for the purpose.

The animals were milked dry immediately before and after the experimental periods. The milk secreted during this time was measured and later analyzed. The blood samples were collected under oil and chilled in ice water immediately. Potassium oxalate was used as an anticoagulant and 10 mg. of sodium fluoride per ml. of blood were used to prevent glycolysis. Lactose in the milk and blood sugar were determined by the method of Somogyi (16).

*Results*

The results of experiments conducted with the technique described above showed that our knowledge of the carbohydrate metabolism of the gland is inadequate. The data from the two experiments shown in Table I indicate that there was considerably more lactose being secreted than could be accounted for

TABLE II

*Levels of Glucose and Lactic Acid in Arterial and Mammary Venous Blood of Lactating Goats*

The results are expressed in mg. per cent.

Glucose			Lactic acid		
Arterial	Venous	Difference	Arterial	Venous	Difference
65.8	50.4	15.4	91.0	80.0	11.0
55.4	41.9	13.5	134.3	76.4	57.9
47.7	37.7	10.0	31.8	10.0	21.8
70.1	49.4	20.7	10.0	7.5	2.5
65.5	41.2	24.3	43.0	14.0	29.0
98.6	80.8	17.8	74.0	42.0	32.0
67.2	48.3	18.9	57.3	51.2	6.1
85.6	52.7	32.9	67.2	41.7	25.5
61.7	35.9	25.8	37.5	24.1	13.4
68.6	40.0	28.6	50.9	41.9	9.0
68.4	47.9	20.5	32.2	27.0	5.0
57.3	42.8	14.5	44.9	28.5	16.4
52.8	39.8	13.0	60.1	50.4	9.7
66.1	51.0	15.1	49.8	41.5	8.3
77.4	49.6	27.8	76.7	58.5	18.2
55.4	38.6	16.8	86.4	69.1	17.3
55.1	40.4	14.7	40.1	40.0	0.1
Average... 65.81	46.96	19.42	58.0	41.4	16.7

by the removal of glucose from the blood and that other carbohydrate-forming compounds must be taken up by the gland.

Accordingly, a second series of experiments was conducted on animals similarly prepared, but without blood flow measurements. Glucose was determined as described above and lactic acid by the Mendel and Goldscheider (17) method. Blood samples were taken as in the first series of experiments.

The results of seventeen such experiments are shown in Table II.

## 4 Lactic Acid Used by Mammary Gland

In these the amount of glucose removed from the blood samples varied from 10 to 32.9 mg. per cent, with an average of 19.4 mg. per cent, or 29.5 per cent of the average level of sugar in the arterial blood.

The method used in the determination of lactic acid in these experiments was chosen because of claims for its specificity. However, since objections might be raised against its use, additional series of blood samples were analyzed for lactic acid by the use of the von Fürth-Charnass technique as modified by West (18). The results of these analyses by the classical oxidation procedure are shown in Table III. The findings were essentially similar to those previously described for animals in lactation. The lower

TABLE III  
*Level of Lactic Acid in Arterial and Mammary Venous Blood*

Arterial	Mammary	Difference	Remarks
<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	
92.1	82.7	9.4	Lactating
103.9	95.0	8.9	
119.8	100.0	19.8	
107.9	97.2	10.7	
81.2	79.8	1.4	
29.7	39.6	-9.9	Non-lactating
21.7	26.5	-4.8	
32.7	47.5	-14.8	
53.5	60.9	-7.4	

average of arterial minus venous differences shown for lactating animals in Table III may be due either to the number of experiments or to the fact that the blood samples were taken later in the lactation period than those shown in Table II. Four experiments on blood from non-lactating animals included in Table III show that the level of lactic acid is higher in the venous than in the arterial blood when the gland is not secreting milk.

Table IV shows the results of two balance experiments carried out essentially in the manner previously described. In the analysis of the blood samples, however, determinations for lactic acid and amino nitrogen were added to those substances previously investigated.

The results show that glucose, lactic acid, and amino nitrogen

are removed from the blood in substantial amounts. 85 per cent of the lactose formed during the experimental period could be accounted for theoretically from the glucose and lactic acid removed from the blood. The remaining 15 per cent may be

TABLE IV

*Results of Two Balance Experiments As Performed in Attempt to Account for Lactose Formed by Mammary Gland As Found in Milk Secreted during Experimental Period*

Experi- ment No.	Duration of experi- ment	Blood analysis						
		Volume flow	Sugar		Lactic acid		Amino N	
			Arterial minus venous	Absolute	Arterial minus venous	Absolute	Arterial minus venous	Absolute
	min.	ml. per min.	mg. per cent	mg.	mg. per cent	mg.	mg. per cent	mg.
26	480	93.3	19.8	934.7	12.7	605.3	1.32	629
28	360	200.7	11.7	855.3	2.3	537.4	0.47	340

*Milk Secreted; Theoretical Production of Lactose\* (from Blood Data)*

Experiment No.	Lactose, absolute	Nitrogen, absolute	From sugar (a)	From lactic acid (b)	Total (a + b)	Lactose accounted for
	mg.	mg.	mg.	mg.	mg.	per cent
26	16,650	3380	8824	5714	14,538	87
28	15,510	1450	8074	5073	13,147	85

*Theoretically Possible Production of Lactose from Amino Acids (Calculated As Alanine)*

Alanine	Lactose from alanine (c)	Theoretical lactose forming total (a + b + c)	Lactose accounted for
mg.	mg.	mg.	per cent
4000	3776	18,314	110
2162	2041	15,188	98

\* Weight of lactose formed = 94.4 per cent of the weight of sugar precursor absorbed.

accounted for if the amino nitrogen removed from the blood is calculated as a 3-carbon amino acid which may be converted into lactic acid.

The validity of calculating amino acid losses from the blood

to the mammary gland as carbohydrate-forming moieties is open to serious question. These calculations as made in Table IV enabled a carbohydrate balance to be found under the conditions of the experiment. There can be little doubt that the blood flow through the vein as measured was well within the possible error of the 15 per cent discrepancy between the theoretical lactose production from glucose plus lactic acid and the actual amount secreted. Recalibration of the flow-measuring elements under various conditions indicates that the method is accurate to within  $\pm 5$  per cent. However, the measurements were made on the mammary vein in these experiments after numerous unsuccessful attempts to measure the arterial flow. While no large efferent vessels remained intact except the one on which measurements were made, there was a small though unknown and unaccounted for amount of peripheral drainage which might lead to a sizable error in calculations. It would, therefore, appear that the results as calculated are probably somewhat low.

Himwich *et al.* (19) have shown that the venous blood from most organs contains a higher level of lactic acid than does the arterial blood. The experimental results shown above are not in accord with such a generalization. These findings place the mammary gland with the heart and the liver in the class of organs in which lactic acid is normally utilized for functional activity.

The experiments show that the withdrawal of glucose alone cannot support the carbohydrate requirements of the heavily secreting mammary gland for the formation of lactose. The average arterial-mammary difference for lactic acid was 16.7 mg. per cent. This figure approaches the difference of 19.4 mg. per cent found for glucose, and augments considerably the carbohydrate uptake of the gland. The results of analyses of blood from non-lactating animals indicate that, under these conditions, lactic acid is produced rather than absorbed by the gland. Therefore, lactic acid may be considered to be an important precursor of lactose in the milk.

The recent work of Gaines (20) has shown that for every pound of lactose secreted by the cow there are 18 pounds of water in the milk. Conditions which result in a decline in the lactose percentage in the milk are usually accompanied by sharp declines in milk volume (21). The interrelations of carbohydrate metab-

olism with phosphorus metabolism are well proved. Milk yields are seriously lowered in phosphorus deficiency (22). The recent work of Bergman and Turner (23) on the hormone stimulation of milk production shows that increases in milk volume in their experimental rabbits were accompanied by greater lactose production. These facts as well as other supporting data point to the production of lactose by the mammary gland as an important factor in the control of milk volume.

Heavy protein feeding, on the other hand, is well known to be a most effective method of increasing milk production. The results of experiments indicate that this protein stimulation of milk production is more effective in the non-ruminant than in the ruminant. Morrison (24), Harrison and Savage (25), and Perkins and Monroe (26) found only small increases in milk secretion after augmenting the protein content of the bovine ration, while Hitchcock (27) and Cox and Mueller (28) present data indicating that lactation in the rat may be greatly augmented by increasing the protein intake of the animals.

The previous experiments of Graham *et al.* (5) have shown that the amount of glucose that is removed from the blood by the lactating mammary gland depends, in a given animal and at a given stage of lactation, on the arterial level of glucose. Since the blood sugar is relatively constant for each species, it cannot be considered to be a flexible source of lactose precursor that could be changed for long periods by feeding. Reactions have been outlined in the work of Symons and Buswell (29) which indicate, as might be expected, that lactic acid would be one of the end-products of the anaerobic fermentation of the complex carbohydrates in the rumen. Such a reservoir to feed the blood with lactic acid is not present in the non-ruminant except possibly in the cecum of Herbivora, thus leaving the strain of lactation in these species on glucose and probably other carbohydrate-forming substances.

Further experiments, as yet unpublished, show that the respiratory quotient of the mammary gland is usually above unity during early lactation. This possible indication of fat formation from carbohydrate might place a further strain on the metabolism of the mammary gland which has not been considered previously. The balance experiments shown in Table IV indicate that amino

## 8 Lactic Acid Used by Mammary Gland

nitrogen could not be the sole precursor of milk nitrogen. Further unpublished experiments indicate that large amounts of protein nitrogen are removed from the blood by the active mammary gland. The amounts of protein removed appear to be sufficient to account for all of the protein nitrogen found in milk. Consequently, the removal of amino nitrogen may or may not be for the purposes of the formation of milk nitrogen.

Recently published experiments (Graham *et al.* (30)) have shown that the mammary gland has the power of producing urea. The mechanism, if working in the manner of our present day conceptions, would furnish an additional supply of carbohydrate to the mammary gland by the deamination of amino acids. This reaction would undoubtedly be of greater importance in the non-ruminant, which would otherwise have to rely on lactic acid from the decomposition of glucose in the blood cells or from diffusion from the muscles, than it is in the ruminant for the reasons already pointed out. It would appear then that the stimulating effects of excess protein feeding on lactation, which are far beyond the effects possibly due to the addition of limiting amino acids, are due *in part* to the ability of the amino acids to furnish excess carbohydrate for the formation of lactose beyond that derived from either lactic acid or glucose.

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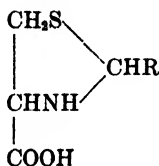
# THE EFFECT OF PYRUVIC ACID ON THE ESTIMATION OF CYSTINE AND CYSTEINE

BY M. X. SULLIVAN AND W. C. HESS

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(Received for publication, August 24, 1937)

In a study of the reaction of aldehydes and ketones with thiol acids Schubert (1, 2) found that cysteine reacts with various aldehydes to form condensation products with the loss of water. The probable structure of the complex formed he gives as



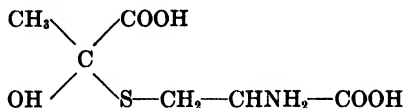
Independently, Ratner and Clarke (3) found that formaldehyde reacts with cysteine to form thiazolidinecarboxylic acid and that formaldehyde and aminoethyl mercaptan give thiazolidine, and described the compounds in detail.

Since aldehydes such as formaldehyde, glyoxal, and methylglyoxal and keto acids such as pyruvic acid are possible metabolic products of proteins, carbohydrates, and fats, and as such might occur more or less in biological solutions, blood and urine, considerable study has been made in this laboratory on their effect on the determination of cystine and cysteine.

In previous work (4, 5) it was shown that in cystine or cysteine determination the various aldehydes have little effect in low molar ratios and dilute solutions. With increasing concentration of cysteine and aldehyde and decreasing acidity, on the other hand, new compounds no longer reacting like cysteine are formed. These compounds, derivatives of thiazolidinecarboxylic acid, described by Schubert and by Ratner and Clarke, do not yield

cysteine readily either by the action of zinc and hydrochloric acid or by dilute acid hydrolysis and are negative in the various reactions for cysteine.

In his early work Schubert (2) considers that cysteine and pyruvic acid, on the other hand, make a simple addition compound with a probable structure of



but in more recent work (personal communication) he questions his early conclusions and considers that the complex between cysteine and pyruvic acid may also be a thiazolidine derivative. Without prejudice as to the nature of the pyruvic acid-cysteine complex, in the present paper we deal with the effect of pyruvic acid on cystine and cysteine determinations by various methods and also with the possibility of liberating cysteine from the complex by simple means.

#### EXPERIMENTAL

*Pyruvic Acid and Cystine*—Solutions of cystine and pyruvic acid in 0.1 N HCl were made so that 5 cc. contained 1 mg. of cystine and increasing amounts of pyruvic acid: (A) 0.3 mg., (B) 3.0 mg., and (C) 5.0 mg. of pyruvic acid. Within 30 minutes of mixing, these solutions were analyzed for cystine by the Sullivan (6), the Okuda (7), and the Folin-Marenzi (8) procedures. The cystine was estimated practically quantitatively by all three methods, even in Solution C, with a molar ratio of pyruvic acid to cystine of approximately 14:1. In fact, with 24 hours contact, 100 per cent of the cystine was recovered colorimetrically in Solution C. Likewise, with relatively concentrated solutions of cystine (1 mg. in 1 cc. of 0.1 N HCl and proportions of pyruvic acid as given above) there was little if any effect on the determination of cystine even in 24 hours standing of the mixture. Thus with 14 moles of pyruvic acid to 1 of cystine, the cystine findings were 97 per cent of the theoretical.

Experiments were also carried out at pH 6.8, the pH of the distilled water, and in phosphate buffer, pH 7.4, the solutions

being so made that each 5 cc. contained 1 mg. of cystine and 0.73 mg. of pyruvic acid (2 moles). After 18 hours standing of the reactants, with the controls respectively in distilled water and buffer at pH 7.4, colorimetric estimation on 5 cc. samples indicated 92 per cent of the theoretical cystine at pH 6.8 and 89 per cent at pH 7.4.

At pH 6.8 and 7.4 the solubility of cystine in the presence of pyruvic acid is questionable, so the results were interpreted to indicate little if any effect of pyruvic acid on the estimation of cystine; in short, no combination such as occurs between cysteine and pyruvic acid, presently detailed.

*Pyruvic Acid and Cysteine, Dilute Solution*—Mixtures were made of cysteine hydrochloride and pyruvic acid so that each 5 cc. contained 1.0 mg. of cysteine and 0.73 mg. of pyruvic acid, that is mole for mole, with the solutions adjusted to pH 1, 2, and 3 respectively. Analyzed by the Sullivan cysteine (9) procedure, at intervals up to 24 hours, the cysteine was recovered quantitatively within the limits of error, at all three pH ranges. The minimum finding of cysteine even after 24 hours standing of the pyruvic acid and cysteine at pH 1, 2, and 3 respectively was 95 per cent of the theoretical. The Folin-Marenzi method run as for cystine showed marked falling off at the end of 6 hours at pH 2.0 (84 per cent return) and at pH 3.0 at the end of 6 hours (80 per cent return).

*Pyruvic Acid and Cysteine, Concentrated Solutions*—Mixtures were made of cysteine hydrochloride and pyruvic acid so that 2.5 cc. of solution contained 20 mg. of cysteine weighed as the hydrochloride and 15 mg. of pyruvic acid, adjusted respectively to pH 1, 2, and 3. Analyzed by the Sullivan method at the end of 6 hours, 70 per cent, 63 per cent, and 59 per cent of the cysteine were recovered at pH 1, 2, and 3 respectively and at 24 hours the results were practically the same. Less reactive cysteine was found by the Folin-Marenzi procedure without sulfite than by the Sullivan procedure. At the end of 2 hours standing the respective percentage recoveries of cysteine at pH 1, 2, and 3 were, Sullivan, 84, 80, and 76; Folin-Marenzi, 75, 60, and 53. The Okuda method without reduction at the end of 24 hours showed 60, 50, and 50 per cent recovery at pH 1, 2, and 3 respectively. However, when the Okuda cystine procedure, reduction with zinc and hydro-

chloric acid, was employed, 100 per cent return of the cysteine was obtained even at the end of 24 hours contact of the reactants at each pH. The Okuda values indicated several possibilities: either the phenomenon was due to greater oxidation of cysteine in the presence of pyruvic acid or the pyruvic acid-cysteine complex was split by the action of zinc and hydrochloric acid or by the acid alone. In order to determine just which of these possibilities was correct the cysteine-pyruvic acid complex was prepared according to the method of Schubert (2).

*Cysteine-Pyruvic Acid Complex*—The crystalline compound melted at 149–151° uncorrected and gave a negative nitroprusside reaction with dilute ammonia, while the addition of aqueous sodium cyanide slowly produced a faint color, as did the addition of dilute sodium hydroxide. For quantitative work 17.2 mg.<sup>1</sup> of the compound were dissolved in 50 cc. of 0.1 N HCl, making a solution equivalent to 200 parts per million of cysteine. The Sullivan cysteine reaction on this solution was very faintly positive. If the cystine reaction was used (2.0 cc. of 5 per cent NaCN in N NaOH, 10 minutes contact) and the solution compared with a 200 parts per million cysteine solution similarly treated, a color was obtained indicating a liberation of 44 per cent of the cysteine. This experiment was repeated but with an increase of the contact time of the sodium cyanide from 10 to 30 minutes. Complete splitting of the complex resulted and a colorimetric estimation of 100 per cent of the cysteine. Substituting 2.0 cc. of N NaOH for the sodium cyanide and giving 30 minutes contact gave a return of 77 per cent of the cysteine. Heating 5 cc. of the pyruvic acid-cysteine complex (equivalent to 1 mg. of cysteine) at 60° for 10 minutes with 2 cc. of 5 per cent NaCN in N NaOH completely liberated the cysteine and gave 100 per cent matching of 5 cc. of cysteine (1 mg.) similarly heated, both being cooled to room temperature before the colorimetric determination. Heating with 2 cc. of 4 N NaOH similarly gave 100 per cent return of the cysteine by the Sullivan cysteine procedure when matched against a cysteine standard similarly treated with both standard and pyruvic acid complex run without any sodium cyanide.

<sup>1</sup> Later work showed the presence of 8.15 per cent potassium chloride, so the actual amount of ash-free material weighed out was 15.8 mg. See "Addendum."

The Okuda method without reduction gave a negative reaction with the cysteine-pyruvic acid product. Application of the Okuda method after reduction with zinc and HCl or simply boiling for 15 minutes with 2 per cent HCl gave 100 per cent return of the cysteine.

The complex can thus be split either by relatively long contact with NaCN and NaOH or by heating with weak acid or relatively strong alkali. By use of 5 per cent NaCN in alkali, cystine, if present, would be estimated and such estimation for the present purpose was undesirable. Accordingly, a more satisfactory procedure for splitting the complex into cysteine and pyruvic acid was used; namely, boiling the material with dilute hydrochloric acid, 2 per cent, and estimating the cysteine by the Sullivan cysteine procedure without cyanide. The procedure used is as follows: 25.9 mg. of cysteine-pyruvic acid complex were dissolved in 75 cc. of distilled water. To 10 cc. was added 0.5 cc. of concentrated HCl and the mixture boiled 10 minutes on the hot-plate, cooled, and neutralized by adding 5 N NaOH dropwise with stirring to pH 3.5 and made to 10 cc. with water. 5 cc. were used for colorimetric work as follows: To 5 cc. add 1 cc. of a 1 per cent aqueous solution of 1,2-naphthoquinone-4-sodium sulfonate, shake for 10 seconds, and add 5 cc. of 10 per cent  $\text{Na}_2\text{SO}_3$  in 0.5 N NaOH, mix, and wait 30 minutes. Then add 1 cc. of 2 per cent  $\text{Na}_2\text{S}_2\text{O}_4$  in 0.5 N NaOH. Match against a cysteine standard (1 mg. in 5 cc. of 0.1 N HCl) similarly treated. By this procedure cysteine only is estimated. A cystine solution containing 1 mg. in 5 cc. of 0.1 N HCl was negative in this procedure. The recovery of cysteine was 95.4 per cent of the theoretical.

*Complete Combining of Pyruvic Acid and Cysteine*—As previously mentioned, cysteine and pyruvic acid, mixed mole for mole and kept for 24 hours at pH 1, 2, and 3, did not become negative in the Sullivan reaction for cysteine. Accordingly the effect of higher pH and higher amounts of pyruvic acid was tried. Thus 20 mg. of cysteine and 15 mg. of pyruvic acid were dissolved in 2.5 cc. of phosphate buffer at pH 6. After 3 hours contact at room temperature, 28–30°, and suitable dilution with 0.1 N HCl for colorimetric work, 59 per cent of the cysteine was found by the Sullivan cysteine method and the same finding obtained after 24 hours contact of the reactants, Solution A. When the pyruvic

acid, however, was increased 10-fold (150 mg.), that is approximately 10 moles of pyruvic acid to 1 of cysteine, practically no cysteine reactive in the Sullivan procedure was left after 1 hour, Solution B.

*Complete Dissociation*—In both cases the cysteine can be readily and completely liberated. Thus 0.3 cc. of each solution was diluted to 10.3 cc. with water and 0.5 cc. of 35 per cent HCl was added and the solutions were boiled for 10 minutes, cooled, neutralized carefully, and diluted to 15 cc. with 0.1 N HCl. When 5 cc. of diluted Solution A were matched colorimetrically against a fresh cysteine standard (1 mg. of cysteine) without use of sodium cyanide, 95 per cent of the theoretical cysteine was found.

In the case of Solution B similarly treated, 96 per cent of the theoretical cysteine was found colorimetrically when matched against a fresh cysteine standard. On the other hand, when the cysteine standard (1 mg. in 5 cc. of buffer at pH 6) was set side by side with the pyruvic acid-cysteine mixture, 101 per cent of the theoretical cysteine was recovered.

For complete combination of cysteine and pyruvic acid, the pyruvic acid must be in considerable excess. The combining reaction is favored by low acidity.

Thiazolidinecarboxylic acid is not dissociated by boiling with dilute acid but can be dissociated by other means.

Preliminary studies of a part day sample of urine tested by the Sullivan and Okuda cysteine methods showed no cysteine in the urine as voided and little if any of a material comparable to the cysteine-pyruvic acid complex. With the cysteine-pyruvic acid compound added to the urine and put through a short heating period with 2 per cent HCl, 94 per cent of the added cysteine in the complex was found by the Sullivan cysteine procedure.

#### SUMMARY

Pyruvic acid added to cystine has little if any effect on the colorimetric estimation of cystine either in dilute or in relatively concentrated solutions and with long contact.

In dilute solutions of cysteine, molar quantities of pyruvic acid have little effect at pH 1, 2, and 3 on cysteine estimation.

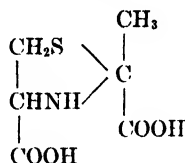
In concentrated solutions the amount of reactive cysteine decreases more rapidly the higher the pH and the greater the

pyruvic acid content, owing to the formation more or less of a non-reactive complex of cysteine and pyruvic acid. At pH 6 the reaction is very rapid.

Unlike the complex formed between cysteine and formaldehyde the pyruvic acid compound is readily dissociated into its components by contact with alkali or acids.

The most satisfactory procedure for dissociating the cysteine-pyruvic acid compound, with practically quantitative yields of cysteine, is by boiling for 10 minutes with 2 per cent HCl.

*Addendum*—Prompted by correspondence with Dr. Schubert, we made an analysis of the cysteine-pyruvic acid complex which by virtue of its N and S content seemed to be an addition product. However, the complex contained 8.15 per cent potassium chloride. Correction for the moisture and ash indicates that the complex is a thiazolidine derivative, as finally concluded by Schubert. The cysteine found colorimetrically is then the cysteine delivered by a substance of the formula



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## A NOTE ON THE DETERMINATION OF POTASSIUM BY THE METHOD OF SHOHL AND BENNETT

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(Received for publication, August 7, 1937)

Shohl and Bennett (1) have adapted the chloroplatinate method to the determination of small amounts (0.1 to 4.0 mg.) of potassium in biological materials. At The Mayo Clinic, by some refinements in technique and careful control of certain variables, we have been able to use this method for the determination of amounts of potassium as small as 0.04 mg.

We have used Hald's (2) method of precipitation. Hald said that porcelain dishes may be used for the ashing procedure. In our laboratory the values obtained for potassium were as much as 10 per cent higher in ashes made in porcelain dishes than they were in ashes made in platinum.

Filter sticks of Jena sintered glass (porosity No. 3) are used for washing and transferring the precipitate. The washed precipitate (completely free from alcohol) is dissolved in a small amount of hot water and transferred by means of slight suction through the filter stick directly into the tube in which the titration is to be carried out.

It is very important in this step to control carefully the amount of water used for the transfer. If the resulting solution is too dilute, low values will be obtained. A concentration of about 0.1 mg. of potassium in 1.5 cc. of water gives most satisfactory results. Values will be about 20 per cent too low if amounts in the range of 0.04 mg. of potassium are determined in a volume of 5 cc.

For this same reason the use of smaller volumes of 4 N potassium iodide is more satisfactory for converting the chloroplatinate to the iodoplatinate than is 2 N potassium iodide. The potassium iodide solution should be prepared immediately before use, since

the presence of traces of free iodine in the potassium iodide will give high results.

A Rehberg (3) microburette<sup>1</sup> which has a capacity of 0.2 cc. is used for the titration.

The iodoplatinate may also be determined colorimetrically, and for the determination of very small amounts this procedure possesses some advantage over the titrimetric procedure. Titration of less than 0.2 mg. is unsatisfactory in laboratories in which no microburette is available. The possibility of adding hydrochloric acid in the colorimetric determination is a distinct advantage. It insures complete development of color even in the presence of very small amounts of  $K_2PtCl_6$ , and traces of alcohol which may remain from the washing procedure do not interfere in the presence of acid. In the colorimetric determination larger

TABLE I  
*Determination of Potassium in Samples of Serum*

Sample	K content of sample	No. of determinations	Average variation from mean	Extremes of variation from mean
	mg.		per cent	per cent
Serum.....	0.214	12	0.8	-0.5 to +1.4
“ + known KCl.....	0.170-0.254	15	1.2	-1.9 “ +1.6
“ .....	0.045-0.435	25	1.9	-3.7 “ +3.7

amounts of water may be used for the transfer of the precipitate; this insures a complete transfer.

Some data obtained with the titrimetric determination on samples of serum are shown in Table I. The method has also been used for determination of potassium in samples of urine, feces, and diets.

I wish to acknowledge with appreciation the advice and suggestions given me throughout the course of this investigation by Dr. E. C. Kendall and Dr. Ancel Keys.

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<sup>1</sup> These microburettes may be obtained from Macalaster-Bicknell Company, Cambridge, Massachusetts.

# A NOTE ON THE USE OF THE HALDANE APPARATUS FOR THE ANALYSIS OF GASES CONTAINING ETHER VAPOR\*

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(Received for publication, September 27, 1937)

It is common knowledge that ether vapor in the ordinary Haldane apparatus prevents the accurate determination of oxygen and carbon dioxide. Ether-free samples analyzed after an ether-laden sample yield results which fall considerably outside the acceptable range of error. To rid the apparatus of ether vapor, the solutions, rubber tubing, and stop-cock grease must be replaced throughout. This note describes the removal of ether from air samples before admission to the Haldane apparatus by a method which does not introduce an error exceeding the range acceptable in ordinary metabolism studies, and which does not entail the replacement of solutions, etc. With this method the oxygen consumption of human subjects or animals may be followed easily and accurately during and after ether anesthesia.

## *Method*

The air to be analyzed is bubbled slowly through concentrated sulfuric acid in a capillary tube before it enters the burette. The apparatus is shown in Fig. 1. Concentrated sulfuric acid is placed in *B* so that the two lower bulbs are half full. A very small drop of mercury in the capillary acts as a valve to reduce the size of the bubbles. The air sample is admitted to the Haldane apparatus by turning *D* to connect the burette and *C*, which is open to *B*. *A* is turned very slowly until the acid begins to bubble at a suitable speed through the acid in *B*. The flow is then controlled

\* This work was made possible by grants from the William F. Milton Fund and the Josiah Macy, Jr., Foundation.

† Research Fellow in Surgery.

## 22 Haldane Analysis of Ether-Laden Gases

entirely by turning *C* as the washing out proceeds. Since *B* and *C* form an extra dead space, six washings with 3 or 4 cc. are recommended to flush the apparatus thoroughly for the first of a pair of determinations; the second may be run after three or four washings. A sample of at least 60 cc. is needed for a duplicate determination. The use of the capillary tube adds about 10 minutes to the time required for a pair of analyses.

Concentrated sulfuric acid dissolves small amounts of oxygen, nitrogen, and carbon dioxide. The acid used in these experiments

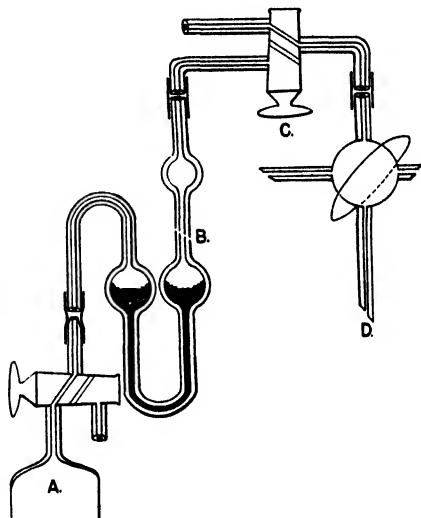


FIG. 1. Haldane apparatus for the analysis of gases containing ether vapor. *A* is the gas-sampling tube. *B* is made of capillary tubing of 1 mm. bore, with two lower bulbs to contain approximately 0.75 cc. and an upper bulb to contain about 0.25 cc. *C* is a two-way stop-cock. *D* is the upper part of the Haldane apparatus (Henderson type stop-cock).

was heated to 100–150° for 15 minutes and stored in a glass-stoppered bottle. Each portion withdrawn for use in the capillary was equilibrated either by rotating it in a small flask for a few minutes with air of approximately the concentration of the sample to be analyzed, or by bubbling a few hundred cc. of the air to be sampled through the acid in the capillary tube.

To determine the effect of sulfuric acid on the percentages of oxygen and carbon dioxide with and without ether vapor, expired

air was collected in a large spirometer in the ordinary manner; after the fan had run for 20 minutes, the air was forced out through a manifold with several jets from which samples were taken in mercury sampling tubes simultaneously and at a uniform rate. Without any change in the set-up, liquid ether was then introduced at the proximal end of the manifold at a rate which yielded from 3 to 6 volumes per cent<sup>1</sup> of ether vapor in the distal end of the manifold; samples were taken as above. Two ether-free samples were analyzed in the ordinary way to determine the composition of the spirometer mixture. One or two ether-free samples were passed through acid and analyzed to note the effect of acid in the absence of ether, and finally, ether-laden samples were passed through the acid. An outdoor air determination was carried out after each set of ether samples to check the apparatus and the efficiency of the acid in removing ether.

### *Results*

Table I contains the data of two experiments with expired air. In neither case is there a significant difference between the oxygen and carbon dioxide percentages of the samples analyzed in the usual manner and those passed through acid.

The average oxygen percentage of the ether-laden samples was slightly less than the ether-free value in both experiments:  $-0.03$  in Sample I, and  $-0.05$  in Sample II, differences which produce 0.2 and 0.3 per cent error in the calculated oxygen consumption.

Correspondingly, the average carbon dioxide percentage in the presence of ether was  $-0.02$  in Sample I, and  $-0.15$  in Sample II, differences which result in 0.6 and 4.6 per cent error in the calculated carbon dioxide production.

Two additional experiments on expired air with and without ether vapor were performed with a different Haldane burette and a different method of exposure of the samples to acid; the results were comparable to those in Table I. The acid was placed in the gas-sampling tube above the mercury. The method is very troublesome since the mercury and sampling tube must be cleaned and freshly set up with new rubber tubing and stop-cock grease every 2 or 3 days. Another procedure was tried—a third chamber

<sup>1</sup> This is approximately the range of concentrations found in the expired air of anesthetized patients (Robbins (1)).

## 24 Haldane Analysis of Ether-Laden Gases

containing sulfuric acid was added to the Haldane apparatus for the absorption of ether, much as in Kruse's technique (2), but

TABLE I  
*Analysis of Expired Air with and without Ether*

Sample No.	Oxygen			Carbon dioxide		
	Standard procedure	Following acid treatment	Ether added followed by acid treatment	Standard procedure	Following acid treatment	Ether added followed by acid treatment
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
I	16.87	16.90	16.82	3.46	3.48	3.43
	16.92	16.88		3.47	3.52	3.48
	16.81	16.93	16.92	3.49	3.48	3.47
	16.91	16.83	16.81	3.49	3.50	3.44
Average	16.88±0.04	16.89±0.03	16.85±0.05	3.48±0.01	3.50±0.02	3.46±0.02
II	17.42	17.39	17.37	3.25	3.25	3.10
	17.38		17.38	3.26		3.12
	17.38		17.30	3.26		3.12
	17.42			3.25		
Average	17.40±0.02	17.39	17.35±0.03	3.26±0.01	3.25	3.11±0.01

TABLE II  
*Analysis of Outdoor Air after Ether Samples*

Date	O <sub>2</sub> + CO <sub>2</sub>
1937	
Mar. 23	20.97
Apr. 11	20.95
June 4	20.96
" 9	20.98
" 11	20.95
" 26	20.98
Average.....	20.97±0.01

with such a chamber outdoor air following an ether sample was variable and usually low; not until the apparatus was set up with fresh solutions could satisfactory readings be obtained.

Table II shows the result obtained on a series of outdoor air samples immediately following analyses of ether-laden samples, carried out by the method described above. The average of the oxygen plus carbon dioxide is 20.97 per cent.

#### SUMMARY

Expired air containing ether vapor may be analyzed with the ordinary Haldane apparatus if a capillary tube charged with concentrated sulfuric acid is inserted between the sampling tube and the Haldane apparatus to remove the ether completely before the air is admitted for analysis. The method requires about 10 minutes more for a duplicate determination of oxygen and carbon dioxide than the usual procedure. The apparatus is ready at once for accurate analysis of ether-free samples without replacement of solutions. Use of acid with samples of air containing from 3 to 6 volumes per cent of ether vapor introduces an error in the calculated oxygen consumption of human subjects of less than 1 per cent and in the calculated carbon dioxide production of less than 5 per cent.

It is a pleasure to thank Dr. A. Baird Hastings and Dr. T. K. Kruse for their suggestions and Miss Murphy and Miss Dewey for their technical assistance.

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# OXIDATION OF CYSTEINE IN NON-AQUEOUS MEDIA\* THE "SULFENIC ACID" AS THE PRIMARY OXIDATION PRODUCT†

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(Received for publication, June 24, 1937)

Since the postulate of Hammett (1930), that oxidation products of the sulfhydryl group are inhibitants of natural growth, has been variously confirmed (Hammett *et al.*, 1936; Zirpolo, 1935; Voegtlin, 1937), it seems important to make available model compounds of any as yet unknown intermediate stages in the oxidation of —SH. That a compound of the type —SOH or —S(O)H may be an important intermediary therein is indicated by the work of many investigators. Mathews (1924) had suggested for the oxidation of cysteine to cystine the mechanism  $R-SH + O \rightarrow R-SOH$  and  $R-SOH + R-SH \rightarrow R-S-S-R + H_2O$ , rather than the conventional formulation  $2R-SH + O \rightarrow R-S-S-R + H_2O$ . Meyerhof (1923) submitted evidence for the existence of a peroxide-like derivative of cysteine, and Dowler (1928) described conditions under which cystine is not oxidized by iodine, while cysteine is oxidized beyond the cystine stage so that the latter can hardly be considered an intermediary in the oxidation of the former, while assumption of the  $R-SOH$  level as the first oxidation stage would be in harmony with the observation. Findings by Brand and associates (1935) as well as by Medes (1937) on the catabolism of cysteine and cystine lend themselves to similar interpretations, and the results of the numerous thermodynamic studies of the reduction potential of cysteine (*cf.* Borsook *et al.* (1937)) also suggest that the actual mechanism of cysteine oxidation may follow a path analogous to the formulation of Mathews rather than that of the single electron transfer  $R-S:H \rightarrow R-S\cdot + \cdot + H^+$  implied in a direct oxidation to cystine.

\* Parts of this investigation have been reported at the meetings of the American Chemical Society, before the Division of Biological Chemistry, at New York, April, 1935, and at Pittsburgh, September, 1936.

† Aided by a grant from the Blanche and Frank Wolf Foundation, Inc.

However, a cysteine derivative of the composition  $R-SOH$  has not been isolated. Simonsen (1933) believed to have evidence for its presence in the products of the oxidation of cysteine with iodine, but did not give any details, and the assumption of an  $-SOH$  compound of relatively high stability by Pirie (1933) has been shown to be erroneous (Toennies, 1937, c). Work of Schöberl and Eck (1936) on amino-free  $-SH$  compounds indicates a strong tendency of the  $-CH_2SOH$  group to turn with loss of  $H_2S$  into an aldehyde group, a conception which is shared by Crowder and Harris (1936) on the basis of their observations on the behavior with alkali of the disulfide group and its oxidized derivatives in wool, while the dismutation of cystine-disulfoxide in alkaline solution (Lavine, 1936) suggests the tendency of the  $-SOH$  derivative of cysteine to react spontaneously according to the equation  $2R-SOH \rightarrow R-SO_2H + R-SH$ .

It is the object of the present paper to render an account of experiments with a method by which cysteine apparently can be oxidized to an  $-SOH$  derivative. Although the goal of isolating the compound has not been attained so far, products have been obtained of which it appears to be, in the form of a sulfate, the main constituent. The method consists in oxidizing cysteine, dissolved as the perchlorate in isoamyl alcohol, with an equimolar amount of permonosulfuric acid in isoamyl alcohol in the presence of an excess of sulfuric acid. The oxidation takes place almost instantaneously, and immediately a white precipitate forms which, according to analytical evidence to be detailed, contains sulfoxycysteine<sup>1</sup> as a sulfate, presumably together with varying amounts of sulfinic and sulfonic acids and of cystine and its disulfoxide.

#### EXPERIMENTAL

*Some Salts of Cysteine and Their Solubilities*—*l*-Cysteine hydrochloride (Merck) and free *l*-cysteine prepared herefrom (Toennies and Bennett, 1935-36) were employed. Ordinary ethyl alcohol

<sup>1</sup> To designate a compound of the composition  $HOOC-CH(NH_2)-CH_2-SOH$  the term sulfoxycysteine, rather than cysteine sulfenic acid, is preferred, as the  $-SOH$  group in the present compound has no noticeable acidic properties. However, no decision as to whether the structure of the group corresponds to  $-SOH$  or  $-S(O)H$  is implied.

(92 per cent) dissolves about 0.5 mole of cysteine hydrochloride per liter, but when 2 mm of free cysteine were treated with an equivalent amount of a 0.217 N solution of sulfuric acid in ethyl alcohol, copious crystallization occurred after initial dissolving; acid equivalent weight 171, calculated for cysteine +  $\text{H}_2\text{SO}_4$ , 170; yield 69 per cent. As the hydrochloride is not suitable for oxidation experiments on account of susceptibility of chloride to oxidation, and as the sulfate is not soluble in alcohol, the perchlorate was investigated. Cysteine dissolved by an equimolar amount of a solution of perchloric acid (68 per cent) in ethyl alcohol formed a relatively stable system in 0.2 M solution:  $[\alpha]_{\text{H}_g} = +6.6^\circ$ , decreasing slowly ( $[\alpha]_{\text{H}_g} = +5.4^\circ$  after 10 days). Solutions of similar stability have been prepared by means of solutions of perchloric acid in isopropyl and isoamyl alcohol as well as in acetic acid. No change in acidity, even after months, appeared in the alcoholic solutions of perchloric acid (0.6 to 0.8 M). Acetic acid solutions seem remarkable for the high rotatory power of cysteine ( $[\alpha]_{\text{H}_g} = +18.5^\circ$ ) and for its stability (the optical rotation of a 0.50 M cysteine perchlorate solution,  $\alpha = +2.23^\circ$  in a 2 dm. tube, remained unchanged during a period of 4 weeks). The unusual stability may be of interest as an expression of the high acidity potential of the perchloric acid-acetic acid system (Hall, 1931).

*Oxidation of Cysteine in Alcoholic Media. Preliminary Experiments*—Studies on solutions of permonosulfuric acid in organic media, described elsewhere (Toennies, 1937, *a*), showed that the peracid reacts relatively slowly with alcoholic —OH groups. In striking contrast herewith is the rapid reaction with the analogous —SH group, shown by the experiments summarized in Table I. The experiments indicate that cysteine takes up 1 atom of active oxygen with great ease, while the reactivity toward a 2nd and a 3rd atom appears to be progressively less. Similar studies on the oxidation of cystine have been previously reported (Toennies, 1934).

Since polarimetric observations under similar conditions indicated the instability of the primary oxidation products—which had also been found in the similar oxidation of cystine (Toennies, 1934)—a search was made for conditions under which the latter might spontaneously precipitate and thus be stabilized. When, instead of ethyl alcohol, isopropyl alcohol or mixtures of isopropyl alcohol

and ether were used as the medium, oxidations with a 1:1 ratio of cysteine and  $\text{H}_2\text{SO}_5$  produced almost immediately small amounts of precipitates consisting apparently of mixtures of intermediary oxidation products in combination with sulfuric acid. Yield as well as composition became more favorable when isoamyl alcohol became the medium. Before detailing the results the general procedure of preparation and the system of analysis will be described. Preliminary to the oxidation the desired amount of finely pulverized cysteine is dissolved by being shaken with the equivalent amount of perchloric acid in a volume of isoamyl

TABLE I  
*Rate of Oxidation of Cysteine by Permonosulfuric Acid in Acid-Alcoholic Medium*

The solvent is 92 per cent ethyl alcohol. To 4 cc. portions, containing 3.3 to 3.7 mm of  $\text{H}_2\text{SO}_4$  and the specified amounts of  $\text{H}_2\text{SO}_5$ , 1 cc. portions of a 0.18 M cysteine perchlorate solution were added. After 55 seconds\* 50 cc. of water containing starch and 1 mm of KI were added and the liberated iodine was titrated at once with 0.024 N  $\text{Na}_2\text{S}_2\text{O}_3$  so that the titrations were completed about 20 seconds later. Each experiment was performed in duplicate.

Molar ratio $\frac{\text{H}_2\text{SO}_5}{\text{Cysteine}}$	$\text{H}_2\text{SO}_5$ reduced per molecule cysteine
	<i>molecules</i>
4.33	2.30, 2.41
3.25	2.18, 2.23
2.17	1.65, 1.59
1.08	1.08, 0.98*

\* In the last experiment the titration was begun after only 25 seconds.

alcohol which will produce about twice the cysteine concentration intended for the oxidation itself. Into this solution, while it is being vigorously shaken, is poured, as rapidly as possible, an approximately equal volume of another isoamyl alcohol solution containing the desired amounts of  $\text{H}_2\text{SO}_5$  and  $\text{H}_2\text{SO}_4$ . Both solutions have been cooled, previous to the reaction, to about  $-10^\circ$ , and the reaction mixture, in a glass-stoppered flask, is shaken for several minutes in a freezing mixture and then at room temperature until the thick milky reaction product, which in most cases begins to form within 5 to 10 seconds after the reactants have been combined, curds into a homogeneous granular precipitate. This

precipitate is strongly acid, water-soluble, and extremely hygroscopic. However, by washing it—on a Buchner funnel in an atmosphere of carbon dioxide (Roeder, 1934) or, more effectively, by digesting, centrifuging, and decanting—with isoamyl alcohol and dry ether (each about six times) and drying in a vacuum desiccator in the presence of  $P_2O_5$  and mineral oil, it is obtained as a loose powder of pure white appearance.

#### *Analytical Methods*

**Acidity**—Suitable samples (e.g. 40 mg.) are titrated directly with 0.05 N NaOH from a microburette, after addition of 1 drop of alcoholic methyl red solution, to the first color change distinguishable within less than 1 minute on rapid titration. The red color returns rather rapidly, and the reaction responsible for this reappearance of acidity is brought to completion by several minutes of boiling. The first end-point, calculated as milliequivalents of acid per 100 mg. of substance, gives "initial acidity" ( $a_i$ ), and the difference between the final and the first end-point, similarly calculated, "formed acidity" ( $a_f$ ).

**Iodine-Reducing and Iodide-Oxidizing Power**—The amount of iodine consumed upon oxidation (presumably to cysteic acid) as well as the amount of iodine liberated upon reduction (to cystine) are determined under conditions previously described (Toennies and Lavine, 1936; Lavine, 1936), suitable cg. quantities being used. The former, expressed as milli-atoms of oxygen (mm of  $I_2$ ) consumed by 100 mg. of substance is termed "reducing value" ( $o^+$ ) and the latter, similarly expressed as oxygen liberated per 100 mg., "oxidizing value" ( $o^-$ ). Since it has been shown (Toennies and Lavine, 1936; Lavine, 1936) that under these analytical conditions cystine and cystine disulfoxide, as well as the sulfinic acid obtained from the latter, are oxidized to cysteic acid, while the disulfoxide and the sulfinic acid, but not cysteic acid, are reduced to cystine, the ratio  $o^+/o^-$  ( $r$ ) may be taken as a measure of the relative level of oxidation (cf. Toennies and Lavine (1933) Table I) of the oxidation product. The ratio  $r$  would be, for cystine  $\infty$ , for the disulfoxide 1.5, for the sulfinic acid 0.667, and for sulfoxycysteine presumably 4.0. Interpretation is, of course, complicated when mixtures of these and other possible intermediate compounds are obtained.

**Sulfur Determinations**—Total sulfur was determined on cg.

quantities by a modification of the  $\text{KMnO}_4$  method of Blix (1928) in which, instead of decomposing the excess  $\text{KMnO}_4$  by  $\text{HCl}$ , it is reduced by methyl alcohol to  $\text{MnO}_2$ , the latter is filtered off and thoroughly washed, and  $\text{BaSO}_4$  is precipitated in the filtrate. The amount of ionizable sulfate present in the reaction products was determined by an adaptation of the volumetric benzidine micromethod of Fiske (1921). The results are again calculated as mm of  $\text{H}_2\text{SO}_4$  per 100 mg., "sulfate content" ( $s_o$ ), and (by difference) "organic sulfur content" ( $s_o$ ) as milli-atoms of organic sulfur per 100 mg. Other analytical criteria used in later stages of the investigation will be described as the occasion arises.

#### *Products Obtained by Oxidation in Isoamyl Alcohol*

A number of preparations obtained and analyzed by the method outlined are summarized in Table II. A comparison of the analytical results with those calculated for some of the possible oxidation products shows that, while mixtures of several of these possibilities are evidently obtained, it seems probable that a sulfate of the composition  $\text{HOOC}-\text{CH}(\text{NH}_2)-\text{CH}_2-\text{SOH} + \text{H}_2\text{SO}_4$  is the chief constituent. Table II further shows that the results of individual experiments are not reproducible within close limits and that when the ratio of oxidant and substrate was deliberately varied (Preparations 13 to 15) erratic variations in the composition of the reaction product resulted. But since the results as a whole suggested that the desired compound, as a sulfate, is formed in substantial amounts, further efforts were directed toward the possibilities of (a) isolating the sulfoxy compound from the mixtures obtained or (b) changing the conditions for the reaction in such a manner that the sulfoxy compound would result in a more nearly pure form.

When the crude precipitates described in Table II were treated with small amounts of 14 M (saturated at  $0^\circ$ )  $\text{HCl}$ , solution with subsequent crystallization occurred. The crystalline precipitates were placed on a porous plate, "washed" by adding single drops of 14 M  $\text{HCl}$ , which was absorbed by the plate, until the substance was nearly free of  $\text{SO}_4^{--}$ , and analyzed after drying *in vacuo* in the presence of a saturated solution of  $\text{NaOH}$ . This treatment furnished supporting evidence for the conclusion that the  $-\text{SOH}$  compound is the main oxidation product, inasmuch as the analyt-

ical values, including those for acidity, of the products obtained by replacement of  $\text{H}_2\text{SO}_4$ , with its two acid groups, by  $\text{HCl}$  were again closely related to those calculated for the corresponding  $\text{R-SOH}$  salt. At the same time no pronounced or reproducible improvement in purity resulted. The best hydrochloride product obtained (by treating 185 mg. of Oxidation Product 7, Table II, at  $-18^\circ$  with 0.70 cc. of 14  $\text{M}$   $\text{HCl}$ ; yield 38 mg.) showed  $a_i = 0.578$ ,  $a_f = 0.187$ ,  $\sigma^+ = 1.235$ ,  $\sigma^- = 0.286$ , and  $r = 4.32$ , compared with the corresponding calculated values of 0.577, 0.191, 1.153, 0.288, and 4.00 for  $\text{R-SOH} + \text{HCl}$ .

#### *Variations in Conditions of Oxidation*

*Reaction Medium*—Attempts to use higher alcohols other than isoamyl did not give promising results. A reaction product obtained in methylisobutyl carbinol was quite similar to those described in Table II; and, in addition, uncomfortable explosive tendencies appeared in solutions of permonosulfuric acid in higher secondary and tertiary alcohols (cf. Toennies (1937, a)). Among other (non-alcoholic) media considered, acetic acid or acetonitrile, while suitable solvents for cysteine perchlorate, did not cause precipitate formation by sulfuric acid upon oxidation; di-*n*-butyl ether dissolves 70 per cent perchloric acid in the presence of 99 per cent sulfuric acid, but the resulting solution does not dissolve cysteine, and a similar result was obtained with di-*n*-butyl sulfate.<sup>2</sup> Evidently the limiting conditions for the suitability of the medium are, apart from non-reactivity, a polarity sufficiently high to permit solution of the salt cysteine perchlorate, and at the same time not high enough to prevent the precipitation of sulfates of the reaction products. In this connection conditions, incidentally discovered, for the formation of an acid sulfate of cystine may be of interest. When 10 cc. of a 0.35  $\text{M}$  cystine perchlorate solution in acetonitrile (Toennies and Lavine, 1933) are carefully combined with 2 to 3 cc. of 15  $\text{M}$   $\text{H}_2\text{SO}_4$ , and a 0.75  $\text{M}$  solution of perchloric acid in isoamyl alcohol is slowly added until a turbidity remains, a crystallization of dense aggregates forms on standing. After thorough washing with ether and drying it showed on titration an acid equivalent weight of 111; calculated for cystine +  $2\text{H}_2\text{SO}_4$ , 109.

<sup>2</sup> A generous sample was kindly supplied by Professor C. Barkenbus of the University of Kentucky.

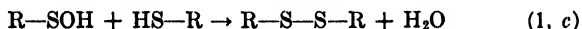
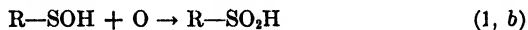


Oxidation No.	Reaction mixture				Reaction products								
	Cysteine perchlorate	Permonosulfuric acid	Ratio (3):(2)	H <sub>2</sub> SO <sub>4</sub>	Cysteine used	Acidity		Reducing value (o <sup>+</sup> )	Oxidizing value (o <sup>-</sup> )	Oxidation-reduction ratio (r)	Organic sulfur content (so)	Sulfate content (so <sub>2</sub> )	Yield by weight of cysteine used
	(2)	(3)	(4)	(5)	(6)	Initial (a <sub>1</sub> )	Formed (a <sub>2</sub> )	(9)	(10)	(11)	(12)	(13)	(14)
(1)						<i>m.-eq. per 100 mg.</i>	<i>m.-eq. per 100 mg.</i>	<i>mili.-atom O per 100 mg.</i>	<i>mili.-atom O per 100 mg.</i>		<i>mili.-atom per 100 mg.</i>	<i>mM per 100 mg.</i>	<i>per cent</i>
5	0.24	0.235	0.98	1.32	1.21	0.784	0.134	0.825	0.252	3.27	0.448	0.383	139
6	0.29	0.275	0.95	1.10	1.09	0.780	0.125	0.825	0.245	3.37	0.464	0.358	119
7	0.255	0.245	0.96	1.23	2.54	0.766	0.156	0.825	0.276	2.99	0.510	0.353	111
8	0.265	0.250	0.96	1.25	4.90	0.755	0.134	0.805	0.261	3.09			107
9	0.265	0.250	0.96	1.20	9.46	0.772	0.129	0.818	0.266	3.08			137
11	0.295	0.280	0.95	2.25	1.81	0.827	0.171	0.777	0.306	2.54			68
13	0.223	0.181	0.81	1.42	1.30			0.662	0.259	2.56			94
14	0.222	0.229	1.03	1.42	1.30			0.742	0.346	2.14			99
15	0.232	0.271	1.17	1.40	1.30			0.928	0.332	2.80			

Calculated values* for	0.851	0.142	0.851	0.213	4.00	0.426	0.426
$\text{HOOC}-\text{CH}(\text{NH}_2)-\text{CH}_2-\text{SOH} + \text{H}_2\text{SO}_4$ .....	0.851	0.142	0.851	0.213	4.00	0.426	0.426
$[\text{HOOC}-\text{CH}(\text{NH}_2)-\text{CH}_2]_2=\text{S}_2 + 2\text{H}_2\text{SO}_4$ .....	0.918	0.000	1.147	0.000	$\infty$	0.459	0.459
$[\text{HOOC}-\text{CH}(\text{NH}_2)-\text{CH}_2]_2=\text{S}_2\text{O} + 2\text{H}_2\text{SO}_4$ .....	0.885	0.148	0.885	0.221	4.00	0.443	0.443
$[\text{HOOC}-\text{CH}(\text{NH}_2)-\text{CH}_2]_2=\text{S}_2\text{O}_2 + 2\text{H}_2\text{SO}_4$ .....	0.855	0.213	0.641	0.427	1.50	0.428	0.428
$\text{HOOC}-\text{CH}(\text{NH}_2)-\text{CH}_2-\text{SO}_2\text{H} + \text{H}_2\text{SO}_4$ .....	1.195	0.000	0.399	0.598	0.67	0.399	0.399
$\text{HOOC}-\text{CH}(\text{NH}_2)-\text{CH}_2-\text{SO}_2\text{H} \dots$ .....	0.654	0.000	0.654	0.981	0.67	0.654	0.000

\* The values in Columns 9 and 10 are based on oxidation to cysteic acid and reduction to cystine respectively. The "formed acidity" values (Column 8) are based, for the disulfoxide, on the experiments of Lavine (1936) who found that in acid solution approximately 1 equivalent of acid is formed from 1 molecule of disulfoxide, and for the  $-\text{SOH}$  compound on the assumed reaction  $3\text{R}-\text{SOH} \rightarrow \text{R}-\text{S}-\text{S}-\text{R} + \text{R}-\text{SO}_2\text{H}$ , i.e. formation of 1 equivalent of acid from 3 molecules of the neutral compound. Additional evidence for this reaction will be given further on. For the monosulfoxide  $[\text{HOOC}-\text{CH}(\text{NH}_2)-\text{CH}_2]_2=\text{S}_2\text{O}$  a primary hydrolysis into  $2\text{R}-\text{SOH}$ , and subsequent reaction of the latter according to  $3\text{R}-\text{SOH} \rightarrow 3\text{R}-\text{S}-\text{S}-\text{R} + \text{R}-\text{SO}_2\text{H}$  is assumed.

*Function of Sulfuric Acid*—An oxidation similar to those of Table II, except that by the use of an  $\text{H}_2\text{SO}_4$  solution of low  $\text{H}_2\text{SO}_4$  content (*cf.* Toennies (1937, *a*)) the  $\text{H}_2\text{SO}_4$  concentration of the reaction mixture was kept at 0.07 M, gave the following result: yield by weight 44 per cent of cysteine,  $a_i = 0.707$ ,  $a_f = 0.011$ ,  $o^+ = 0.693$ ,  $o^- = 0.090$ ,  $r = 7.7$ ,  $so_4 = 0.250$ , and  $ss$  (disulfide) = 0.12. The latter value was obtained by Shinohara's modification of Folin's phosphotungstic acid method (Shinohara, 1935-36), on the assumption—later confirmed (see below)—that  $\text{R-SOH}$  in the presence of bisulfite reacts rapidly according to  $\text{R-SOH} + \text{NaHSO}_3 \rightarrow \text{R-S-SO}_3\text{Na} + \text{H}_2\text{O}$ . The amount of  $\text{R-SOH}$  obtained in this oxidation is obviously small (*cf.*  $a_f$ ,  $o^-$ , and  $r$ ; a calculated composition of 0.205 mm of  $\text{H}_2\text{SO}_4$ , 0.11 mm of cystine, 0.03 mm of  $\text{R-SOH}$ , 0.05 mm of  $\text{R-SO}_2\text{H}$ , and 0.25 mm of  $\text{R-SO}_3\text{H}$  accounts reasonably well for the analytical data), indicating that the presence of sulfuric acid has a profound effect on the amount of  $\text{R-SOH}$  intercepted. On the basis of this and the preceding experiments the following equations may serve as a tentative picture of what happens in the oxidations.



and further analogous oxygen additions which would lead to cysteic acid, disulfoxides, etc. But also

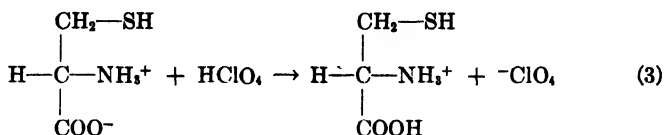


and similarly for cystine and the other oxidation products, presumably excluding those of an acid nature, as the sulfinic and cysteic acids, which, if formed, may be expected to precipitate as such.

Several implications are inherent to this conception. If Equation 1, *a* represents the primary reaction, success in obtaining its product as the insoluble sulfate depends on the competitive situation between the reaction of Equations 1, *b*, 1, *c*, 1, *d*, etc., on the one hand and the reaction of Equation 2 on the other. Therefore, lowering of the concentration of  $\text{R-SH}$  and  $\text{H}_2\text{SO}_4$  (main-

taining their ratio at unity) while keeping the concentration of  $\text{H}_2\text{SO}_4$  constant should favor the reaction of Equation 2 at the expense of the reactions of Equations 1, b, etc., and the same effect should result from increasing the  $\text{H}_2\text{SO}_4$  concentration while the concentrations of substrate and oxidant remain constant.

*Bisulfates in Place of Sulfuric Acid*—Furthermore, inasmuch as cysteine, etc. are present in solution as -onium ions, held in this state by the acid function of perchloric acid,



the actual reaction which leads to the sulfate precipitates may be thought of as one of the -onium ions with the bisulfate ion,



rather than with  $\text{H}_2\text{SO}_4$  molecules, as suggested by Equation 2. Since  $\text{H}_2\text{SO}_4$ , as a relatively weak acid compared with  $\text{HClO}_4$  (cf. e.g. Hall (1931)), is presumably incompletely ionized, even in its first acid group, in a medium of low dielectric constant such as isoamyl alcohol, substitution of a soluble bisulfate for the free acid could be expected to favor the rapidity of the precipitation reaction indicated by Equation 4. It was surprising to find that when the cysteine oxidation was carried out under conditions where most of the sulfuric acid was replaced by monoamylamine bisulfate<sup>3</sup> (0.245 M cysteine perchlorate, 0.25 M  $\text{H}_2\text{SO}_5$ , 0.09 M  $\text{H}_2\text{SO}_4$ , and 1.50 M  $\text{C}_5\text{H}_{11}\text{NH}_3^+ ^-\text{SO}_4\text{H}$ ) no precipitation whatever

<sup>3</sup> Monoamylamine bisulfate was easily prepared by adding the amine (Sharples Solvent Corporation, 96 per cent according to titration) slowly to an equimolar amount of 99 per cent  $\text{H}_2\text{SO}_4$  frozen in freezing mixture. After it was heated on the steam bath in order to dissolve some lumpy material (the neutral sulfate, which is insoluble in the base or in amyl alcohol), a clear and almost colorless syrup resulted which had a density of about 1.22 and contained 6.24 milli-equivalents of  $^-\text{HSO}_4$  per cc. according to titration of an aqueous dilution. The total acid content obtained by titration after the amine was expelled by boiling with excess alkali corresponded to 13.04 milli-equivalents per cc. and was equal to that calculated from the total  $\text{H}_2\text{SO}_4$  used. This syrupy substance is miscible with such solvents as amyl alcohol or acetonitrile.

occurred. Another experiment, in which monobutylamine bisulfate was used (0.17 M cysteine perchlorate, 0.17 M  $\text{H}_2\text{SO}_5$ , 0.27 M  $\text{H}_2\text{SO}_4$ , 1.00 M  $\text{C}_4\text{H}_9\text{NH}_3^+\text{-SO}_4\text{H}$ ), likewise gave no precipitate whatsoever in spite of the relatively high concentration of free  $\text{H}_2\text{SO}_4$ . For lack of actual knowledge about the state of ionization and dissociation of  $\text{H}_2\text{SO}_4$  and its salts in isoamyl alcohol no explanation, but merely the hypothesis of complex formation being involved, can be advanced to account for this unexpected result. That the presence of the amine in itself does not prevent the precipitate formation is indicated by the appearance of a precipitate upon addition, to the reaction mixture of the above reported oxidation in the presence of amylamine sulfate, of an amount of 0.8 M  $\text{HClO}_4$  in isoamyl alcohol equivalent to the amount of bisulfate present. Thus apparently free  $\text{H}_2\text{SO}_4$ , reformed from the bisulfate by the action of the stronger  $\text{HClO}_4$ , is necessary for the precipitation to occur. Also there is no evidence that an oxidation of the amine (present in the  $\text{NH}_3^+$  form, see below under "Evidence for identity of sulfoxycysteine") by the peracid could interfere with the oxidation of the cysteine by the latter.<sup>4</sup>

*Phosphoric and Permonophosphoric Acids*—Since  $\text{H}_3\text{PO}_4$  showed a precipitate-forming action similar to that of  $\text{H}_2\text{SO}_4$ , it was necessary to ascertain whether or not results would justify its substitution for  $\text{H}_2\text{SO}_4$ . In order not to have two different precipitating agents present, *viz.*  $\text{H}_3\text{PO}_4$  and  $\text{H}_2\text{SO}_4$  resulting from  $\text{H}_2\text{SO}_5$ , the latter was replaced by permonophosphoric acid,  $\text{H}_3\text{PO}_5$ . The concentrations were 0.23 M cysteine perchlorate, 0.23 M  $\text{H}_3\text{PO}_5$ , 1.15 M  $\text{H}_3\text{PO}_4$ , and the medium isoamyl alcohol containing 10 per cent acetonitrile, introduced with the permonophosphoric acid (Toennies, 1937, b). The heat of reaction was distinctly greater than in the corresponding  $\text{H}_2\text{SO}_5$  experiments and precipitation began within 2 seconds. The precipitate (yield 50 per cent of

<sup>4</sup> Rather was it found that the amyl alcoholic solution of the peracid is rendered more stable by the presence of the amine sulfate. At room temperature a solution of 0.075 M  $\text{H}_2\text{SO}_5$  and 0.055 M  $\text{H}_2\text{SO}_4$  showed, after 2 and after 24 hours, decreases in peracid (*cf.* Toennies (1937, a)) of 22 and 95 per cent respectively, while the same solution, containing in addition  $\text{C}_4\text{H}_9\text{NH}_3^+\text{-SO}_4\text{H}$  in 0.53 M concentration, showed decreases of only 17 and 53 per cent over the same periods.

weight of cysteine) gave the following data,  $a_i = 0.535$ ,  $a_f = 0.000$ ,  $o^+ = 0.523$ ,  $o^- = 0.031$ ,  $r = 16.9$ ,  $po_4 = 0.254$ , and  $ss = 0.100$ , which suggest absence of R—SOH (no  $a_f$ , cf. foot-note to Table II) and formation of large proportions of cystine and cysteic acid. This result, which is in sharp contrast with those of the analogous  $H_2SO_5$ — $H_2SO_4$  experiments, may be explained either by the higher oxidizing power of  $H_3PO_5$  (indicated by the heat of reaction) which causes the oxidation to pass rapidly beyond the R—SOH stage, or by a lower precipitation affinity of  $H_3PO_4$ . That the latter is a factor is suggested by the outcome of another experiment in which  $H_2SO_5$  was the oxidant and  $H_3PO_4$  the quantitatively predominating precipitant. The concentrations used were 0.25 M cysteine perchlorate, 0.25 M  $H_2SO_5$ , 1.24 M  $H_3PO_4$ , and 0.07 M  $H_2SO_4$ ; and the result, yield 40 per cent of weight of cysteine,  $a_i = 0.665$ ,  $a_f = 0.000$ ,  $o^+ = 0.727$ ,  $o^- = 0.041$ ,  $r = 17.7$ ,  $so_4 = 0.198$ ,  $po_4 = 0.065$ , and  $ss = 0.138$ , indicating not only that again cystine and cysteic acid are the prevailing products, but also that  $H_2SO_4$  has stronger precipitating tendencies than  $H_3PO_4$ , since it predominates in the precipitate even though its total concentration (0.25 M from  $H_2SO_5$  and 0.07 M free  $H_2SO_4$ ) amounted only to 26 per cent of that of  $H_3PO_4$ .

*Variations in Concentrations*—When the superiority of  $H_2SO_4$  and  $H_2SO_5$  over the corresponding phosphorus compounds had thus become evident, the effect of changes in the ratio precipitant to substrate-oxidant, discussed in a previous paragraph ("Function of sulfuric acid"), was investigated. However, lowering the substrate-oxidant concentration to one-tenth of its former value (0.025 M), while sulfuric acid remains 1.25 M, apparently makes the whole system too dilute, since no precipitate appeared in the oxidation. Even when the medium at these concentrations was 10 per cent isoamyl alcohol and 90 per cent ether—a mixture in which cysteine perchlorate remains soluble—only a small amount of precipitate was formed. It thus remained to see what the effect would be of increasing the ratio of precipitant to substrate-oxidant by an increase of the sulfuric acid concentration. Solutions of 4 M  $H_2SO_4$  in isoamyl alcohol could be obtained by running the  $H_2SO_4$  (99 per cent) very slowly (about 0.4 cc. per minute) into the alcohol which was maintained at less than 0°. The resulting solution is of oily consistency and, even at low temperature, subject

TABLE III

*Oxidation of Cysteine in Isoamyl Alcohol in Presence of 4 M Sulfuric Acid*

Of the symbols not previously explained  $nh_2$  means amino nitrogen according to Van Slyke, factor 0.926 being used;  $ss$ , disulfide according to Shinohara (1935-36), the dry substance being dissolved in the bisulfite solution;  $ss_f$ , additional disulfide, formed spontaneously in the aqueous solution of the substance;  $sh^+$  maximal amount of cysteine which reacts with the substance;  $a^-$  acid liberated in the reaction with excess cysteine. The technique and interpretation of these determinations are discussed in the text.

Reaction	Oxidation Product 34		Oxidation Product 35	
Cysteine, $HClO_4$ , $M$ .....	0.104		0.099	
$H_2SO_4$ , $M$ .....	0.106		0.098	
$H_2SO_4$ , ".....	4.0		4.0	
Volume, cc.....	38.5		107	
Temperature, $^{\circ}C$ .....	About $-10$		$-5$	
Precipitate	Found	Calculated	Found	Calculated
Yield, by weight, per cent.....	87		86	
$so_4$ , $mM$ .....	0.387	0.387	0.389	0.389
$s_o$ , milli-atom.....			0.447	0.457
$nh_2$ , $mM$ .....			0.451	0.457
$a_1$ , $m.-eq$ .....	0.802	0.800	0.800	0.825
$a_f$ , ".....	0.133	0.133	0.123	0.123
$ss_1$ , $mM$ .....	0.050	0.050	0.042	0.042
$ss_f$ , ".....	0.092	0.092	0.094	0.094
$o^+$ , milli-atom.....	0.904	0.890	0.915	0.866
$o^-$ , ".....	0.250	0.270	0.248	0.278
$r$ .....	3.62	3.30	3.69	3.11
$sh^+$ , $mM$ .....	0.276	0.276	0.276	0.283
$a^-$ , $m.-eq$ .....			0.042	0.043

Calculated composition

	$mM$ per 100 $mg$ .	$mM$ per 100 $mg$ .
$H_2SO_4$ .....	0.387	0.389
Cystine.....	0.050	0.042
Sulfoxycysteine.....	0.214	0.240
Cystine disulfoxide.....	0.062	0.043
Cysteine sulfinic acid.....	0.026	0.047

to esterification and gradual darkening. However, since shortly after the preparation titration showed the calculated acidity, the solutions were used soon after they were obtained. For the oxidations both halves (substrate and oxidant) were made of approximately equal  $\text{H}_2\text{SO}_4$  concentration, by addition of the required amount of  $\text{H}_2\text{SO}_4$  to the cysteine perchlorate solution in isoamyl alcohol, while in the other half  $\text{H}_2\text{SO}_6$  in concentrated solution was added to the previously prepared  $\text{H}_2\text{SO}_4$ -isoamyl alcohol mixture. Two experiments of this type were performed, both at  $-5^\circ$  to  $-10^\circ$ ; one in which the solutions were mixed as usual and one in which the  $\text{H}_2\text{SO}_6$  solution was rapidly emptied into the cysteine solution which was being stirred at high speed (about 800 R.P.M.). The latter method was tried as it was thought that uneven distribution, due to imperfect mixing of the viscous solutions, might cause local deviations from the 1:1 ratio between substrate and oxidant and thus favor the formation of those compounds which are higher and lower in the oxidation scale than the sulfoxo compound. The results of the two experiments are summarized in Table III. They indicate in the first place that rapid stirring has no profound effect on the nature of the reaction product. Secondly, they show markedly different analytical values from those of Table II. Whether or not the higher ratio precipitant to oxidant-substrate has resulted in a better yield of the desired compound cannot be stated with certainty as not all of the analytical criteria reported in Table III were applied to the earlier products. Since, for the time being, additional experimentation on the problem could not be undertaken, the work was brought to a temporary conclusion by expanding the scope of analytical tests applied to the oxidation products and finally putting the question of the presence of sulfoxycysteine to a crucial test.

#### *Evidence for Identity of Sulfoxycysteine*

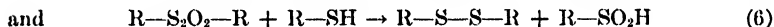
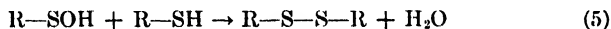
The amino N determination on Oxidation Product 35 (Table III) shows substantial equivalence between  $-\text{NH}_2$  groups and organic sulfur and thus supports the conclusion (Toennies, 1934) that the amino group in its acid form is protected against oxida-



tion by the absence of free electron pairs<sup>5</sup> ( $\text{R}:\ddot{\text{N}}:\text{H}$ , while the

basic form  $\text{R}:\ddot{\text{N}}:$  is susceptible to addition of oxygen,  $\text{R}:\ddot{\text{N}}:\text{O}$  and subsequent changes).

If the gradual formation of cystine and acidity which occurs in solution is due to spontaneous changes of the compound  $\text{R}-\text{SOH}$ , acid groups and cystine molecules may be expected to appear in equal numbers (cf. foot-note to Table II). Fig. 1 shows that the ratio of  $a_f$  and  $ss_f$  is not unity but approximately 1.31 in the case under investigation. If the higher ratio is tentatively attributed to the presence of cystine disulfoxide—which in acid solution forms acid groups and cystine molecules in a 3:1 ratio (Lavine, 1936)—the calculated values for sulfoxycysteine and cystine disulfoxide of Table III result. If these are correct the postulated reactions



require the calculated values given in Table III under  $sh^+$  and  $a^-$  for the amounts of cysteine consumed and acid simultaneously liberated. The cysteine consumption was determined by dissolving weighed samples corresponding to a calculated content of 0.07 to 0.08 mm of  $-\text{SOH} + -\text{S}_2\text{O}_2-$  in 25 cc. flasks with freshly standardized neutral cysteine solution corresponding to 0.10 mm of  $-\text{SH}$ . The remaining  $-\text{SH}$  was determined on 4 cc. portions by the technique of Shinohara (1935). The amount of cysteine found consumed, calculated per 100 mg. of substance, ( $sh^+$ ) was 0.284, 0.270, and 0.275 mm in three independent experiments and the amount of acid liberated in the reaction ( $a^-$ ), obtained by titration and the subtraction of the value of  $a_i$  (0.800), was 0.038, 0.050, and 0.042 milli-equivalent. Thus the results are in substantial agreement with the amounts of  $-\text{SOH}$  and  $-\text{S}_2\text{O}_2-$

<sup>5</sup> Just as ammonia in the  $\text{H}:\ddot{\text{N}}:\text{H}$  form is stable under oxidizing conditions in the Kjeldahl method.

calculated from the spontaneously formed amounts of cystine and acid. However, it was noted that while in a comparable experiment with cystine disulfoxide the maximal amount of cysteine

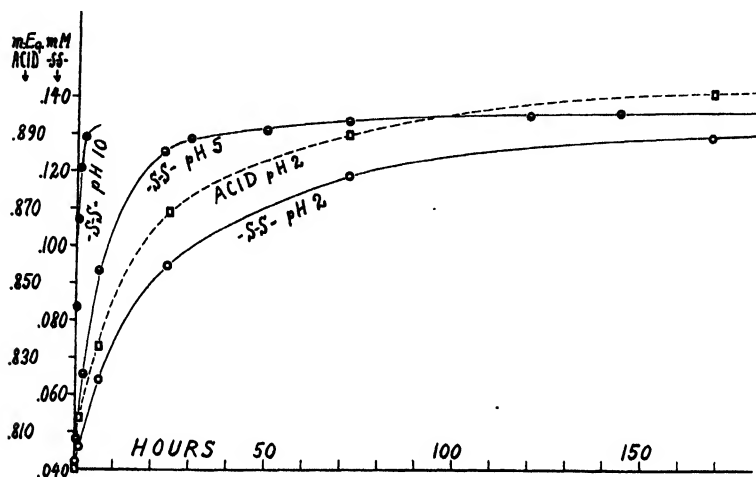


FIG. 1. The spontaneous formation of cystine and acidity from an oxidation product in solution. 106.2 mg. of Oxidation Product 35 (Table III) were dissolved to 100 cc. with water, and periodical determinations were made of  $-S-S-$  by the Folin-Shinohara method (Shinohara, 1935-36), with 4 cc. samples made up to 25 cc. for the determination, and of the acidity by titrating 10 cc. samples with 0.05 *N* NaOH from a microburette to the intermediate gray color of brom-cresol purple (pH 6). The two resulting curves are labeled "acid pH 2" and " $-S-S-$  pH 2" (the acidity of this solution was about pH 2.3 in the beginning). The curves " $-S-S-$  pH 5" and " $-S-S-$  pH 10" are the result of determinations made on solutions of similar concentrations which contained 20 mm of sodium acetate and 6 mm of acetic acid (pH 5.2) and 1.263 milli-equivalents of NaOH per 100 mg. (pH 10) respectively. The time of determination for the  $-S-S-$  values was defined as the moment of addition of the bisulfite, on the assumption—borne out by the constancy of the values obtained—that the spontaneous changes are arrested by the action of the bisulfite,  $R-S_2O_2-R + NaHSO_3 \rightarrow R-S-SO_3Na + R-SO_2H$  (Lavine, 1936) and presumably  $R-SOH + NaHSO_3 \rightarrow R-S-SO_3Na + H_2O$ .

had reacted 2 minutes after its addition, in the case of the present substance the final value was not attained until after about  $\frac{1}{2}$  hour, the reaction being only about 80 per cent complete after 2 minutes.

As a further consequence and final means of verification of the

assigned composition may be taken the amount of cystine resulting from the reaction expressed by Equations 5 and 6. To this end 127.8 mg. of Oxidation Product 35 and 43.6 mg. of cysteine were dissolved in 20 cc. of water previously brought to the sensitive intermediate gray shade (pH 4.9) of a 3:2 mixture of brom-cresol blue and methyl red (Kolthoff, 1929). Within 25 minutes a total of 1.080 mm of NaOH was added to neutralize the acid liberated in the reaction. After 24 hours standing in the refrigerator, the pH (4.9) remaining unchanged, 61.35 mg. of cystine were obtained by filtration and drying. It was identified by its optical rotation in a 0.5 per cent solution in *N* HCl. The value found  $[\alpha]_{\text{H}_g}^{31.2} = -231^\circ \pm 1^\circ$ , compared with the one calculated for pure *l*-cystine (Andrews, 1925; Toennies and Lavine, 1930),  $[\alpha]_{\text{H}_g}^{31.2} = -235^\circ$ , indicates a purity of the cystine precipitate of 98 per cent. The filtrate was made up to 100 cc. The amount of iodine liberated (*cf.* "Methods") by 20 cc. fractions was 0.0236, 0.0257 mm. The filter paper on which the crystallized cystine had been collected was treated with 5 cc. of *N* H<sub>2</sub>SO<sub>4</sub> in order to dissolve residual cystine. The acid washings, made up to 50 cc., contained, according to a determination with phosphotungstic acid (Shinohara, 1935-36) 0.0304 mm of disulfide, while the filtrate itself by the same method showed 0.142 mm of disulfide. In order further to identify this portion 50 cc. of the filtrate were evaporated to dryness, leaving a residue, dried over P<sub>2</sub>O<sub>5</sub>, of 62.9 mg. The solution of this residue in *N* HCl (total volume 6.70 cc.) showed  $\alpha_{\text{H}_g}^{31} = -0.49^\circ$  (1 dm. tube). These analytical data are the basis of the following computations, which are referred to the unit of 100 mg. of substance. The calculated values are based on the analytical data given in Table III.

Cysteine used for reaction.....	0.282 mm
"    calculated to be required.....	0.283 "
Acidity, after reaction with cysteine.....	0.845 m.-eq.
"    before " " " " .....	0.800 "
"    increase, found.....	0.045 "
"    "    calculated.....	0.043 "
Iodide-oxidizing power, before reaction with cysteine.....	0.248 mm I <sub>2</sub>
Iodide-oxidizing power, after reaction with cysteine.....	0.096 " "
Iodide-oxidizing power, decrease, found .....	0.152 " "
"    "    "    calculated.....	0.142 " "

## Evaporation residue of filtrate

Na <sub>2</sub> SO <sub>4</sub> , calculated.....	0.389 mm = 55.2 mg.
Cystine, colorimetrically.....	0.111 " = 26.8 "
R-SO <sub>2</sub> Na from R-S <sub>2</sub> O <sub>2</sub> -R, calculated.	0.043 " = 7.5 "
"    "    original assumed	
R-SO <sub>2</sub> H.....	0.047 " = 8.2 "
Calculated.....	97.7 "
Found.....	98.4 "

Assuming the value  $[\alpha]_{H_g} = -33^\circ$  (Lavine, 1936) for the specific rotation of the sulfinic acid and assuming that the amount of the latter present in the evaporation residue lies between (per 100 mg. of substance) 0.090 mm (according to original calculation) and 0.043 mm (resulting from disulfoxide only), one obtains a calculated correction of  $+0.03^\circ \pm 0.01^\circ$  and, therefore, the rotation to be assigned to cystine as  $= -0.52^\circ \pm 0.01^\circ$ , or a value for its specific rotation of  $[\alpha]_{H_g}^{21} = -0.52^\circ \times 6.70/0.017 = -205^\circ$ , compared with a calculated value of  $-235^\circ$ . On this basis the minimum amount of *l*-cystine present would be 87 per cent of the colorimetric value, *i.e.* 0.097 mm (per 100 mg.).

*l*-Cystine identified

Main portion, according to weight and optical rotation..	0.204 <sup>mm</sup>
"    "    residue on filter, colorimetrically.....	0.024
Filtrate, colorimetrically 0.111 mm } mean value.....	0.104
"    polarimetrically 0.097 " }	
Total, after reaction with cysteine.....	0.332
Found (colorimetrically) before reaction with cysteine...	0.042
Increase, found.....	0.290
"    calculated.....	0.283

The result of this experiment confirms the assumed composition as far as R-S-S-R, R-S<sub>2</sub>O<sub>2</sub>-R, and R-SOH are concerned, since the values obtained for the changes in acidity, iodide-oxidizing power, and cystine are substantially within the range of the calculated ones. Uncertainty must be admitted regarding the fraction designated as R-SO<sub>2</sub>H in the original calculation (Table III), on account of the considerable discrepancies between the calculated and found values for  $\alpha_i$ ,  $\sigma^-$ , and  $\sigma^+$ . Numerous observations encountered in the course of this work point toward the possible existence of a cysteine derivative R-SO<sub>2</sub>H, isomeric with the sulfinic acid which results from alkaline decomposition of the disulfoxide (Lavine, 1936), but having neither acid- nor iodide-

oxidizing properties; a compound which also may be involved in the acid decomposition of the disulfoxide (Lavine, 1936). However, less than 10 per cent by weight of the product under investigation is involved in this uncertainty since 93 per cent (including  $\text{H}_2\text{SO}_4$ ) may be considered identified as *l*-cystine or oxidation products of *l*-cysteine-cystine which by combining with *l*-cysteine are converted into *l*-cystine. The largest part of this cystine-forming fraction combines with cysteine without liberation of acid; a condition which, together with the other analytical data, is satisfied by the level of oxidation that corresponds to the addition of 1 oxygen atom to the cysteine molecule. Whether the compound is present in its anhydride form ( $\text{R}-\text{S}_2\text{O}-\text{R}$ , cystine monosulfoxide) or as sulfoxycysteine ( $\text{R}-\text{S}(\text{O})\text{H}$  or  $\text{R}-\text{SOH}$ ) cannot be definitely stated, although the latter form is favored by the analytical values and seems by far the most likely according to the expected ease of its formation in the interaction of equimolar amounts of cysteine and peracid.

#### SUMMARY

The possibility of oxidizing cysteine to the sulfenic acid level by rapid oxidation in non-aqueous media has been studied. Permonosulfuric acid acting in isoamyl alcohol upon cysteine perchlorate causes immediate precipitation of sulfate-containing products in which the desired compound appears to be present in varying amounts. The effect of experimental variations has been studied and the identity of the chief oxidation product as a cysteine derivative corresponding to the sulfenic acid level has been established by a quantitative demonstration of the reaction with cysteine which conforms with the equation  $\text{R}-\text{SOH} + \text{R}-\text{SH} \rightarrow \text{R}-\text{S}-\text{S}-\text{R} + \text{H}_2\text{O}$ .

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## THE CARBOHYDRATE METABOLISM OF BRAIN

### V. THE EFFECT OF CERTAIN NARCOTICS AND CONVULSANT DRUGS UPON THE CARBOHYDRATE AND PHOSPHO-CREATINE CONTENT OF RABBIT BRAIN\*

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Asher and Takahashi (1) concluded that "convulsions which are due either wholly or partially to excitation of the central nervous system are capable of diminishing the carbohydrate store of the central nervous system." This conclusion was based on experiments with insulin, strychnine, and picrotoxin (2) with the use of a method for glycogen determination which the authors admitted included galactose from cerebrosides.<sup>1</sup>

With a method for glycogen determination designed to avoid postmortem glycolysis as well as contamination with cerebrosides (4) it was recently shown in this laboratory that insulin causes a decrease in the glycogen of brain even in the absence of convulsions (5). It was considered advisable to test further the conclusions of Asher and Takahashi (1) concerning the effects of drugs which cause convulsions by excitation of the central nervous system. Accordingly we have studied the effects of strychnine, picrotoxin, and cardiazole in convulsant doses, but are unable to confirm the findings of the Berne workers. Since our technique of freezing the brain *in vivo* requires the use of an anesthetic, we were obliged to study also the effects of anesthesia, and used this opportunity to test the findings of Uchida (6) on the effect

\* Aided by a grant from the Rockefeller Foundation.

<sup>1</sup> Although a modified method which they believed to be more accurate gave results only a third as great, no repetition of the experiments was reported. They adhered, however, to their conclusions (1, 3) because evidence was found that the material estimated by their procedure contained glycogen.



of ether and chloroform narcosis on the glycogen and free sugar of brain. Although the results in respect to brain glycogen are negative in both groups of experiments, we believe them to be of sufficient interest to justify publication in summarized form.

Rabbits which had fasted for 18 to 24 hours before experimentation were used. The brains were frozen *in situ* and analyzed by the methods used in previous studies of this series (5).

TABLE I

*Effect of Certain Narcotics and Convulsant Drugs on Carbohydrate, Lactic Acid, and Phosphocreatine of Rabbit Cerebrum*

Remarks	Duration of anesthesia	No. of animals	Blood sugar, mg. per 100 cc.		Mg. per 100 gm. cerebrum							
					Phospho-creatine P		Glyco-gen		Free sugar		Lactic acid	
					Mean	$\sigma$ *	Mean	$\sigma$	Mean	$\sigma$	Mean	$\sigma$
	min.											
Amytal.....	9-14	8	95	15	11.6†	0.5	85	17	75	18	22	12
Evipan.....	4-8½	8	138	10	12.2	0.9	89	11	65	14	15	5
Ether.....	2½-5	8	153	9	11.3	1.5	91	15	72	15	24	6
".....	60	6			11.8	1.5	105	13	126	22	25	8
Chloroform.....	2½-5	8	134	25	10.7	1.5	98	18	60	16	41	18
".....	30	4			11.8	1.7	94	9	102	20	28	5
Picrotoxin (amytal)	9-14	10	172	64	9.6	1.2	75	31	66	36	22	14
" (ether)...	2½-5	4			11.0	0.8	84	6	84	18	33	8
Cardiazole (amytal)	9-14	9	195	53	9.9	0.8	72	17	88	26	40	13
" (ether)...	2½-5	4			11.2	0.4	103	21	88	29	35	5
Strychnine (amytal)	9-14	6	156	19			96	18	93	13	21	6

\* The standard deviation was calculated from the formula,  $\sigma = \sqrt{\Sigma x^2/n}$ .

† Determined on three rabbits not of this series.

*Effects of Narcosis*—Amytal (sodium salt) and evipan were administered intravenously, the dosage for amytal being 80 mg. and for evipan 60 to 250 mg. per kilo of body weight. Ether was given by inhalation in concentration sufficient to produce deep surgical anesthesia. For one group of eight animals the brain was frozen as soon as anesthesia was complete (2½ to 5 minutes), while for a second group the period was prolonged to 1 hour. With chloroform also experiments were made, both with a short and a long period of anesthesia. The results, summarized in

Table I, show no significant variation in glycogen or phosphocreatine values. With evipan the minimum value for lactic acid was obtained, together with a maximum value for phosphocreatine, and a free sugar level similar to that previously reported for control animals under amytal anesthesia (5, 7).

Results with amytal confirm those previously reported (5, 7), although the levels of lactic acid and free sugar are slightly raised. With both ether and chloroform, neither short nor long periods of anesthesia produced any significant change other than a raised level of free sugar in brain after an hour of anesthesia<sup>2</sup> and an increase in lactic acid a few minutes after narcosis with chloroform. Our findings therefore give no support to those of Uchida (6), who reported marked decreases in brain glycogen and free sugar during ether and chloroform anesthesia.

Our results are of interest in view of the findings of Emerson (8), who noted a decreased rate of "autoxidation" in the excised brains of rats previously anesthetized with ether or chloroform for an hour. Emerson explained this on the basis of Uchida's results (*i.e.* a lowered carbohydrate content), which we are unable to confirm. It should also be noted that the carbohydrates of brain are almost completely converted to lactic acid within the period required to carry out an experiment on the respiration of excised brain (9).

*Effect of Drugs Producing Convulsions by Stimulation of Central Nervous System*—The drugs used (strychnine, picrotoxin, and cardiazole) were injected subcutaneously at intervals of 30 to 60 minutes until convulsions occurred. With strychnine one to four doses of 5 to 10 mg. each were required. With picrotoxin three to seven doses of 10 to 20 mg. (over a period of 6 hours) were needed, whereas single doses of 30 mg. produced convulsions within 30 minutes. One or two doses of cardiazole (100 to 200 mg. total) caused convulsions within 10 to 90 minutes. The animals were anesthetized with amytal as soon as convulsions occurred in some cases, whereas in others the convulsions were allowed to continue for 5 or 10 minutes.

Since changes produced in the brain during or before convulsions might be reversed during the interval of 10 or more minutes re-

<sup>2</sup> The amount of free sugar in brain fluctuates to a certain extent according to the level in blood (5).

quired for completion of anesthesia with amytal, the experiments were repeated with ether as anesthetic. This permitted the beginning of freezing of the brain within  $1\frac{1}{2}$  to 3 minutes.

The results (Table I) do not confirm those of Asher and Takahashi (2), for no decrease in glycogen of the brain could be consistently produced with either picrotoxin or strychnine. Glycogen values after picrotoxin convulsions showed wide fluctuations with amytal (but not with ether), two of the values being below the normal limits and one above, but in eleven of the fourteen experiments the glycogen level was within the range of the controls. The decrease in the glycogen average is too small to be considered significant.

With strychnine and cardiazole also no consistent or significant lowering of glycogen was observed.

Some increase of free sugar in brain is noted after convulsions, particularly with strychnine.<sup>2</sup> A slight increase in the amount of lactic acid is found in brain after convulsions resulting from cardiazole, and also after picrotoxin with ether as anesthetic. No increase is found with picrotoxin or strychnine under amytal anesthesia.

No alteration in the phosphocreatine content of brain occurs as the result of the experimental procedures which caused convulsions.

We conclude that excitation of the central nervous system to an extent sufficient to cause convulsions is not regularly associated with any significant change in the carbohydrate fractions studied nor in the phosphocreatine content of brain.

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## NOTE ON THE PHOSPHORUS CONTENT OF RAT BRAIN IN EXPERIMENTAL RICKETS

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(Received for publication, August 24, 1937)

Hess, Gross, Weinstock, and Berliner (1) reported a lowered content of inorganic phosphate and of calcium in rat brain as a result of experimental rickets. Since subsequent work (2) showed that organic phosphorus compounds present in brain decompose with great rapidity after death, it became necessary to restudy the effect of rickets.

Rickets was produced in albino rats (20 to 30 days old) by feeding the Steenbock Ration 2965 (3), while the litter mate controls were given the same diet with an added 2 per cent of cod liver oil. After moderate or severe rickets had been demonstrated by x-ray examination in all the animals on the rachitogenic diet, the rats were anesthetized with amytal (100 mg. per kilo of body weight) and the brains frozen *in situ* with liquid air. The preparation of the specimen for analysis and the methods of determination of some of the phosphorus fractions are described elsewhere (4). The amount of organic phosphorus hydrolyzed in the presence of  $N$  HCl at  $100^{\circ}$  in 15 minutes obviously cannot be assigned to any one compound, especially since the nature of most of the organic phosphorus of brain remains unknown. Purine nucleotide was determined by the method of Kerr and Blish (5). For the determination of total phosphorus the material was digested with concentrated  $HNO_3$ , evaporated to dryness, and then ignited with sodium carbonate-nitrate mixture, after which the phosphorus was determined colorimetrically (6).

The results (Table I) show that phosphocreatine and the acid-insoluble (lipoid) phosphorus fraction are consistently lower in all the rachitic brains as compared with the controls, but the

difference is small and of doubtful significance. In the other fractions no significant differences between the normal and rachitic brains are found.

TABLE I  
*Phosphorus Fractions of Brain in Normal and Rachitic Rats*

Experiment No.	Remarks	No. of rats	Days on special ration	Average weight at end of experiment	Degree of rickets indicated by x-ray	Mg. per 100 gm. cerebrum							
						Phosphocreatine P	Inorganic P	Organic P hydrolyzed in 15 min.	Undetermined acid-soluble P	Total acid-soluble P	Acid-insoluble P	Total P	Nucleotide N
				gm.									
25-3	Normal	7	70	127	0	11.3	28.3	16.1	30.1	85.8	251	337	25.1
	"	7	70		0	11.3	24.4	15.8	32.4	83.9	254	338	23.6
	Rachitic	6	70	124	+++	10.7	29.2	16.1	33.4	89.4	236	325	29.1
25-4	Normal	7	33	82	0	11.1	36.3	17.2	28.4	93.0	216	309	28.3
	"	7	33	75	0	11.4	40.0	18.6	31.6	101.6	232	334	29.5
	"	7	33	70	0	11.4	33.2	19.7	26.9	91.2	233	324	28.3
	Rachitic	9	33	61	++	8.5	22.9	21.3	29.2	81.9	208	290	26.3
	"	9	33	72	++	10.6	23.3	23.6	28.7	87.2	Lost	Lost	
	"	9	33	63	++	9.2	18.0	21.1	29.7	78.0	222	300	29.9
25-7	Normal	7	56	100	0	10.9	27.9	17.5	29.9	86.2	255	341	28.0
	Rachitic	5	56	89	++	8.5	32.4	17.6	32.2	90.7	237	328	29.0
Mean for normal.....						11.2	31.7	17.5	29.9	90.3	240	331	27.1
$\sigma^*$ for normal.....						0.2	5.4	1.4	1.8	6.0	15	11	2.1
Mean for rachitic.....						9.5	25.2	19.9	30.6	85.4	226	311	28.6
$\sigma^*$ for rachitic.....						1.0	5.0	2.7	1.9	4.8	12	16	1.4

\* The standard deviation was calculated from the formula,  $\sigma = \sqrt{\sum x^2/n}$ .

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## STUDIES ON THE MERCAPTURIC ACID SYNTHESIS IN ANIMALS

### VII. BROMOBENZENE AND *l*-CYSTINE IN RELATION TO GROWTH OF RATS ON A NAVY BEAN MEAL DIET

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(Received for publication, August 18, 1937)

Haley and Samuelsen (1) recently reported some experiments the results of which indicated that bromobenzene had no consistent, if any, effect on the growth of rats which were maintained on the cooked navy bean meal diet of Johns and Finks (2). The data of Johns and Finks (2) and those of Osborne and Mendel<sup>1</sup> indicated, however, that the navy bean meal is deficient in *l*-cystine inasmuch as considerably better growth was obtained on the navy bean meal diet supplemented with *l*-cystine than on the navy bean meal diet alone. In the light of recent experiments by Rose *et al.* (3) and by Beach and White (4), these experiments further showed that the navy bean meal was adequate in its methionine content. The finding of Haley and Samuelsen (1) appeared surprising, therefore, particularly because White and Jackson (5) have demonstrated that bromobenzene checks the growth of rats receiving a casein diet which was adequate for moderate growth but low in organic sulfur, and that the growth is resumed upon supplementing the bromobenzene diet with *l*-cystine or methionine.

#### EXPERIMENTAL

The diet which we used had the following percentage composition: navy bean meal<sup>2</sup> 71, salt mixture (Osborne and Mendel (6)) 4, butter 15, and lard 10. In addition, each rat received 400 mg. of

<sup>1</sup> The experiments of Osborne and Mendel were incorporated in the paper of Johns and Finks (2).

<sup>2</sup> The navy beans were boiled for 3 hours in distilled water, evaporated to dryness on a steam bath, and powdered.

yeast powder (Northwestern Yeast Company) and 100 mg. of cod liver oil daily. Johns and Finks (2) and Haley and Samuelsen (1) employed a similar diet, omitting yeast and cod liver oil.

The general procedure of the experiments was the same as that used previously (5, 7). The data shown in Table I are representa-

TABLE I

*Effect of Bromobenzene on Growth of Rats Maintained 10 Days on Navy Bean Meal Diet*

The data shown in this table are representative of experiments with fourteen rats.

Rat No.	Diet*	Food intake per day	Initial weight	Total gain in 10 days
		gm.	gm.	gm.
14 ♂	N-B-71	6.0	44	+17
	N-B-71-B	6.6	61	+6
	N-B-71†	6.6	67	+25
15 ♂	N-B-71	9.9	56	+19
	N-B-71-B	7.0	75	+1
	N-B-71†	7.0	76	+25
16 ♂	N-B-71	6.3	49	+18
	N-B-71-B	5.5	67	+4
	N-B-71-B with cystine	8.2	71	+38
17 ♂	N-B-71	6.1	54	+16
	N-B-71-B	6.0	70	+1
	N-B-71-B with cystine	10.0	71	+48
18 ♂	N-B-71	6.4	54	+15
	N-B-71-B	5.7	69	+7
	N-B-71-B with cystine	8.2	76	+40
19 ♂	N-B-71	7.2	43	+17
	N-B-71	11.4	60	+23
	N-B-71	9.7	83	+28

\* Diet N-B-71 denotes the navy bean meal diet; Diet N-B-71-B, the navy bean meal diet with 1.0 gm. of bromobenzene per 100 gm. of the diet.

† The food intake was restricted to that consumed by the same rat while receiving bromobenzene in the diet.

tive of the experiments obtained with two litters of albino rats which were 26 days old.

The *l*-cystine was analytically pure and the bromobenzene was redistilled before use.

#### DISCUSSION

The data presented in Table I indicate that the growth of rats ceased when bromobenzene was incorporated in the navy bean

meal diet and was resumed when each 100 gm. of the diet was supplemented with 120 mg. of *l*-cystine. The stimulation of growth by *l*-cystine indicates that the diet was ample in its methionine content. Our results are not in accord with those reported by Haley and Samuelsen (1).

Under the conditions of our experiments, the navy bean diet apparently lacked sufficient organic sulfur to meet the requirements for growth and the detoxication of bromobenzene. The results emphasize the conclusion made previously (5, 8) that, in the growing animal, the detoxication of bromobenzene takes precedence over the need for organic sulfur for growth purposes.

It is of interest to note that the rats of Haley and Samuelsen (1) grew at a considerably greater rate on the navy bean meal diet than on the same diet supplemented with *l*-cystine. These results are somewhat surprising, since the experiments of Johns and Finks and Osborne and Mendel (2) have conclusively demonstrated that the navy bean meal diet is deficient in cystine.

#### SUMMARY

1. The growth of rats is checked when bromobenzene is added to a navy bean meal diet and is resumed when the diet containing bromobenzene is supplemented with *l*-cystine. These results are not in accord with those of Haley and Samuelsen (1).

2. In the light of experiments by Rose *et al.* (3) and Beach and White (4), the results suggest that the navy bean meal diet is ample in its methionine content.

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## THE DIFFUSIBLE CALCIUM OF SERUM AND TRANSUDATES IN VIVO\*

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A large body of evidence has accumulated to indicate that part of the calcium present in the blood serum is non-diffusible, probably largely non-ionized, and bound to the serum proteins. The present work attempts to test this theory and to estimate the magnitude of the diffusible and non-diffusible fractions by comparisons of the quantities of calcium and protein in serum and transudates from the same subjects.

Evidence that part of the calcium in serum is combined with protein in a non-diffusible form has been derived by a variety of methods.<sup>1</sup> Loeb (4) has shown that in solutions of serum proteins the diffusibility of calcium varies with the concentration of protein. Compensation dialysis or ultrafiltration has revealed that about 40 to 60 per cent of the calcium of serum, depending upon the conditions of the experiments, is freely diffusible (5-11). Compensation dialysis has generally yielded higher results than ultrafiltration. However, Greene and Power (12) by conducting dialysis *in vivo* secured values more nearly in agreement with those obtained by ultrafiltration.

Through statistical analyses of large numbers of sera from patients without manifest disturbances of calcification Hastings, Murray, and Sendroy (13) and Peters and Eiserson (14) developed

\* This article represents work done in fulfillment of the thesis requirement for the degree of Doctor of Medicine at Yale University School of Medicine.

<sup>1</sup> The literature pertaining to this subject has been discussed in more detail by Peters and Van Slyke (1), Thomson and Collip (2), and Schmidt and Greenberg (3).

an empirical equation connecting serum calcium with protein, when inorganic phosphorus was within normal limits,  $\text{Ca} = 0.556 \text{ protein} + 6$ , in which calcium concentration is expressed in mg. per cent and protein in gm. per cent. Other equations of a similar nature, but with different constants, have been derived by numerous observers (15-17). The correlation obviously depends upon the fact that diffusible calcium is held within a narrow range by physiological regulation. The intercept with the calcium axis represents the mean concentration of diffusible calcium, while the slope represents mg. of Ca bound per gm. of protein. Estimations by this method of the amount of calcium bound by protein can be only rough approximations, because of the multiplicity of factors which may affect the total calcium of serum.

Ultrafiltration and compensation dialysis leave something to be desired, since there can be no certainty that conditions which obtain *in vivo* can be reduplicated with accuracy *in vitro*. For this reason solution of the problem has been sought in comparisons of sera with various body fluids, such as spinal fluid (18, 19) and lymph (20) and with transudates (21). Spinal fluid has proved unsatisfactory, since it has been established that large variations of serum calcium are not reflected by comparable changes in the spinal fluid (19). The calcium of both thoracic duct lymph (20) and of transudates (21) is distinctly lower than that of the serum and varies with the latter when this is altered by injections of parathyroid extract and by parathyroidectomy, procedures which presumably alter the diffusible calcium of serum, since they do not alter the concentration of protein. Furthermore it has been established that the pH (22, 23) and inorganic phosphorus (21, 24, 25) of sera and transudates are approximately the same, a matter of no little importance, since both pH and inorganic phosphorus appear to have some influence upon the concentration of calcium in the serum (1, 14, 26-28). Materials used for these comparisons of sera and transudates have not been properly selected to permit evaluation of the calcium-combining power of total protein or of the albumin and globulin fractions in serum.

### *Procedures*

For statistical purposes 331 routine analyses of serum for calcium and protein have been employed. The sera were treated by the methods described below.

The material for comparison of serum and transudates was obtained from twenty-five patients. Observations 1, 2, and 3 were made on the same subject; Nos. 13 and 14, 18 and 19, and 22 and 23 represent duplicate observations on three different patients. In two instances, Observations 4 and 5, material was obtained after death: heart's blood, pleural, ascitic, and edema fluid were taken within 15 minutes from Patient 5, and heart's blood and pleural fluid within an hour from Patient 4.<sup>2</sup> With these exceptions all examinations were made while the subjects were in the postabsorptive state.

Seven of the patients were suffering from congestive heart failure, complicating either arteriosclerotic or rheumatic heart disease; one had adhesive pericarditis; three had portal, and one, biliary cirrhosis of the liver; three had acute and two chronic nephritis; two glomerulonephritis in the nephrotic stage. Patient 4, with congenital absence of the right kidney, and a calculus in the left ureter, developed complete ureteral obstruction, anuria, uremia, edema, and died shortly after admission. Besides diabetes Patient 22 had both congestive heart failure and a nephrotic syndrome. One patient had mercury poisoning, another presumably malnutritional edema, although his serum proteins were 5.66 per cent; the last two had diabetes with staphylococcus septicemia.

Blood and fluid (ascitic or pleural) were collected simultaneously. Blood, withdrawn without stasis, after it had clotted, was centrifuged for 20 minutes, when the serum was separated. The whole procedure was conducted with anaerobic precautions. Fluids that clotted or became cloudy were also centrifuged. Serum and fluid were then stored in the refrigerator until they were analyzed. A small amount of whole blood treated with oxalate was used for determination of non-protein nitrogen.

Inorganic phosphorus was determined by the method of Benedict and Theis (29) in Observations 1 to 14 and 25 to 28 and in the 331 routine analyses, by the method of Fiske and Subbarow (30) in Nos. 15 to 23 and 30, and by a micromodification of the Fiske and Subbarow procedure in Nos. 24 and 29. In every case the

<sup>2</sup> The use of postmortem fluid appears to be justifiable, as inorganic phosphorus increases equally in serum and transudates after death (unpublished data from this laboratory).

TABLE I  
Analyses of Serum and Transudates

Observation No.	Fluid	Protein	Albumin	Globulin	Ca	Inorganic P	Diagnosis and remarks
		gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	
1	Serum	6.67	4.37	2.30	12.93	2.60	Diabetes, nephrosclerosis
	Pleural	2.06	1.62	0.44	12.25	2.89	
2	Serum	6.36	4.42	1.94	9.67	3.25	Same, 10 days later
	Pleural	2.29	1.85	0.44	7.56	3.57	
3	Serum	6.45	4.22	2.23	12.95	3.12	" 46 " "
	Pleural	2.38	1.82	0.56	10.20	3.12	
4	Serum	5.19	3.60	1.59	9.80	16.34	Absent right kidney; left ureteral calculus. Collected 1 hr. after death
	Pleural	3.19	2.14	1.05	8.04	15.00	
5	Serum	4.06	2.35	1.71	6.24	10.10	Nephrosclerosis. Serum and all fluids collected 15 min. after death
	Pleural	1.37	0.86	0.51	4.63	10.67	
5, a	Ascitic	0.68			4.00	10.87	
5, b	Edema	0.37			4.34	11.12	
6	Serum	6.39			13.20	3.08	Biliary cirrhosis. Icteric index 75
	Ascitic	1.11			6.47	3.23	
7	Serum	5.14	2.76	2.38	8.40	6.56	Acute nephritis
	Pleural	0.93	0.63	0.30	6.17	6.29	
8	Serum	6.35	3.47	2.88	9.80	3.61	Rheumatic heart disease; pleurisy
	Pleural	4.81	2.75	2.06	8.50	3.78	
9	Serum	5.66	2.91	2.75	9.11	3.78	Hemachromatosis; diabetes
	Pleural	0.44	0.21	0.23	6.36	3.41	
10	Serum	5.19	3.51	1.68	9.57	4.12	Acute nephritis
	Pleural	0.96	0.74	0.22	6.85	4.12	
11	Serum	5.60	2.36	3.24	8.82	2.49	Diabetes, portal cirrhosis
	Ascitic	1.30	0.63	0.67	6.33	2.40	
12	Serum	7.24	3.86	3.38	10.10	4.12	Syphilitic heart disease
	Pleural	4.66	2.68	1.98	5.10	3.49	
13	Serum	5.21	2.70	2.51	9.06	5.36	Same, 1 day later
	Ascitic	2.48	1.48	1.00	7.41	5.36	
14	Serum	4.28	2.47	1.81	7.37	4.00	Adhesive pericarditis; malnutrition
	Ascitic	1.29	0.92	0.37	6.18	4.28	
15	Serum	5.12	3.23	1.89	8.80	4.46	Acute nephritis
	Pleural	1.07	0.53	0.54	6.80	4.17	
16	Serum	5.97	3.38	2.59	9.76	2.98	Diabetes; secondary anemia
	Pleural	1.18	0.77	0.41	6.81	3.00	
17	Serum	4.60	2.66	1.94	8.12	22.30	Chronic glomerular nephritis
	Pleural	2.68	1.58	1.10	6.21	25.90	

TABLE I—*Concluded*

Observation No.	Fluid	Protein	Albumin	Globulin	Ca	Inorganic P	Diagnosis and remarks
		gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	
18	Serum	<b>5.96</b>	<b>3.70</b>	<b>2.26</b>	<b>6.17</b>	<b>7.26</b>	Rheumatic heart disease; chronic nephritis; pneumonia
	Pleural	1.29	1.04	0.25	4.40	7.53	
19	Serum	<b>4.57</b>	<b>2.80</b>	<b>1.77</b>	<b>5.88</b>	<b>10.52</b>	Same, 1 day later
	Pleural	1.43	0.92	0.51	4.14	10.89	
20	Serum	<b>3.42</b>	<b>2.00</b>	<b>1.42</b>	<b>8.63</b>	<b>7.12</b>	Glomerular nephritis; nephrotic stage
	Pleural	0.31			6.62	6.86	
21	Serum	<b>3.83</b>	<b>2.22</b>	<b>1.61</b>	<b>5.05</b>	<b>16.41</b>	Chronic nephritis; pneumonia. 2 hrs. between collection of serum and fluid
	Pleural	2.29	1.27	1.02	4.27	13.80	
22	Serum	<b>4.27</b>	<b>2.40</b>	<b>1.87</b>	<b>7.82</b>	<b>6.06</b>	Diabetes; arteriosclerotic heart disease
	Pleural	0.77	0.46	0.31	5.91	2.77	
23	Serum	<b>2.76</b>	<b>1.45</b>	<b>1.31</b>	<b>12.84</b>	<b>4.52</b>	Same, 9 days later after acacia
	Pleural	1.25	0.69	0.56	8.61	4.76	
24	Serum	<b>5.40</b>	<b>3.52</b>	<b>1.88</b>	<b>8.52</b>	<b>5.18</b>	Mercury poisoning
	Ascitic	1.75	1.17	0.58	6.69	6.26	
25	Serum	<b>7.26</b>	<b>3.97</b>	<b>3.29</b>	<b>9.93</b>	<b>3.83</b>	Arteriosclerotic heart disease
	Ascitic	4.11	2.39	1.72	8.02	3.00	
26	Serum	<b>6.16</b>	<b>2.79</b>	<b>3.37</b>	<b>8.06</b>	<b>3.85</b>	Portal cirrhosis. Icteric index 100
	Ascitic	1.24	0.58	0.66	5.95	4.57	
27	Serum	<b>6.15</b>	<b>2.99</b>	<b>3.16</b>	<b>9.30</b>	<b>4.91</b>	Portal cirrhosis, chronic nephritis
	Ascitic	1.53	0.77	0.76	7.15	5.36	
28	Serum	<b>6.03</b>	<b>2.67</b>	<b>3.36</b>	<b>8.44</b>	<b>3.72</b>	Diabetes with acidosis; serous pleurisy; acute sialadenitis
	Pleural	2.87	1.30	1.57	6.54	3.94	
29	Serum	<b>3.04</b>	<b>1.32</b>	<b>1.72</b>	<b>6.41</b>	<b>7.02</b>	Nephrosis
	Ascitic	0.11	0.06	0.05	4.74	7.10	
30	Serum	<b>3.49</b>	<b>1.94</b>	<b>1.55</b>	<b>13.10</b>		Diabetes; staphylococcus septicemia; after acacia
	Pleural	1.65	1.08	0.57	7.02	1.70	

same method was applied to both serum and fluid. All analyses were made within 12 hours after collection of the blood and fluid.

Total nitrogen was determined by the macro-Kjeldahl procedure; the nitrogen of protein fractions by the method of Howe (31). For the latter 1 cc. of serum and 2 cc. of fluid were used. The fractions were precipitated within 12 hours.

Calcium was determined by the Clark and Collip (32) modification of the method of Kramer and Tisdall in Observations 1 to 13

and 25 to 27 and in the 331 routine analyses. In the remaining cases serum was ashed and analyzed by the procedure described by Hald (33). In every instance analyses were made in duplicate.

### Results

From the 331 routine analyses, by the usual mathematical method, the following regression equation was obtained,  $\text{Ca} = 0.66 \text{ protein} + 5.4$ .

The results of the analyses of serum and fluids are given in Table I. The differences between the concentrations in serum and fluid of the various constituents will be represented by the following symbols: protein,  $\Delta\text{Pr}$ , albumin,  $\Delta\text{A}$ , globulin,  $\Delta\text{G}$ , calcium,  $\Delta\text{Ca}$ , phosphorus,  $\Delta\text{P}$ . Protein values are expressed in gm. per cent, calcium and phosphorus in mg. per cent. The concentration of Ca is always lower in fluid than in serum. On the other hand P is higher in serum ten times, lower eighteen times, and the same in fluid and serum three times. In twenty-three of the thirty-two observations the differences do not exceed the experimental errors of the methods employed. The mean  $\Delta\text{P}$  is  $-0.03$  mg. per cent, which agrees closely with values obtained by other observers (21, 24, 25). The influence of inorganic phosphate on calcium should, then, be the same in serum as in fluid and can hardly be responsible for the differences in the concentrations of calcium in the two media.

In Fig. 1, in which  $\Delta\text{Ca}$  is plotted against  $\Delta\text{Pr}$ , twenty-seven of the thirty-two observations tend to fall along a straight line. Of the remaining five, two represent analyses of sera after the administration of acacia. Since acacia is known to contain calcium in non-diffusible combination (34) and to remain in the blood stream for a considerable period, an explanation is afforded for these two discrepancies. Between Observations 22 and 23 which are from the same case serum Ca rose, after acacia, from 7.82 to 12.84 mg. per cent. In Patient 30 no observations are available before administration of acacia. Afterwards Ca in serum was found to be 13.10, in fluid 7.02 mg. per cent. The concentrations in both serum and fluid are distinctly above normal, although the patient had no other evidences of a disorder of calcification. Determination, by weight, of the solids in serum and fluid gave evidence of enough acacia in both to account for the increases of calcium.

In the other three instances, no such satisfactory explanation can be found for the lack of correlation between calcium and protein. In Observation 1 pleural fluid Ca was disproportionately high. Observations 2 and 3 from the same patient at short intervals thereafter were consistent with the majority of the results, although the serum Ca in No. 3 was as high as it was in No. 1. Although this suggests an analytical error, there is no direct evidence of error in the experiment. Patient 6 proved at autopsy to have biliary cirrhosis with intrahepatic pericholangitis.

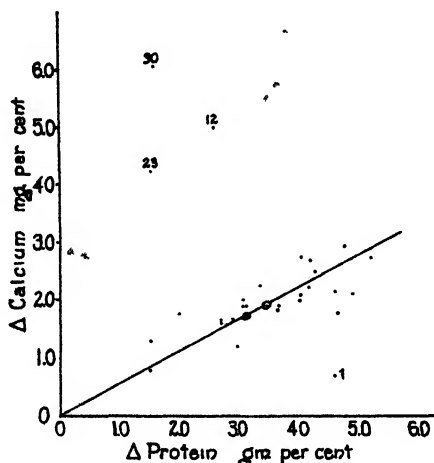


FIG. 1. The relation of calcium to protein in serum and transudates.  $\Delta\text{Ca}$  and  $\Delta\text{Pr}$  represent respectively the differences between the concentrations of calcium and protein in serum and transudates. The small figures opposite divergent points correspond to the numbers of these observations in Table I.

Patient 12 had syphilitic carditis with moderate congestive failure. The divergences from the normal relationship between calcium and protein in these last three observations are so great that it must be presumed that either the combining power of protein was peculiar or that some other unknown factor is responsible.

#### *Analysis of Results*

If the difference between Ca of serum and fluids depends upon the combination of calcium with protein in a non-diffusible form,



as numerous observers have suggested (21, 24, 35, 36),  $\Delta\text{Ca}$  should vary directly with  $\Delta\text{Pr}$ . With the exception of the five divergent observations that were discussed above, the existence of such a direct correlation is evident from Fig. 1. The correlation coefficient between  $\Delta\text{Ca}$  and  $\Delta\text{Pr}$  is  $0.62 \pm 0.08$ . If only the observations following acacia, Nos. 23 and 30, are omitted, the correlation coefficient,  $0.37 \pm 0.11$ , is still statistically significant. If the relation between  $\Delta\text{Ca}$  and  $\Delta\text{Pr}$  is linear, as Fig. 1 suggests, and Ca is bound quantitatively by protein, either in chemical combination or by adsorption, the line describing this relation should be a straight line, passing through the origin. The equation which best fits the data and conforms to these requirements, derived in the usual manner (to make the sum of the squares of the residuals minimum), is  $\Delta\text{Ca} = 0.561 \Delta\text{Pr}$ .<sup>3</sup>

The standard deviation of this equation is 0.372 mg. per cent and the probable error  $\pm 0.25$ . The error of measuring Ca is  $\pm 0.2$  mg. and the error of estimating  $\Delta\text{Ca}$  may be twice as great. In addition there is the possibility, even under the carefully standardized conditions of these experiments, that exact equilibrium between serum and fluid may not be established. In view of all these facts and possibilities the correlation must be considered more than satisfactory. It should be noticed how closely the slope of this equation agrees with that derived by Peters and Eiserson (14),  $\text{Ca} = 0.566 \text{Pr} + 6$ .

Since protein fractions were determined in both serum and fluid in twenty-four cases, the relative combining powers of albumin and globulin were also estimated by similar methods. It was assumed that both albumin and globulin combined with calcium in regular proportions. That is,  $\Delta\text{Ca} = K_1\Delta\text{A} + K_2\Delta\text{G}$ .

The equation of this form which fits the data best proved to be  $\Delta\text{Ca} = 0.639 \Delta\text{A} + 0.435 \Delta\text{G}$ .

#### DISCUSSION

Loeb (4), in 1926, showed that serum globulin altered the diffusibility of calcium through collodion membranes. In 1924, Csapó

<sup>3</sup> In the derivation of this equation and subsequent treatment of the data the five aberrant observations, Nos. 1, 6, 12, 23, and 30, have been omitted. This is mathematically justified in the three experiments without acacia, because the deviation of Point 1 is 5 times and the deviations of Points 6 and 12 are 10 times as great as the standard deviation.

and Fauble (37) found that, when albumin and globulin were precipitated, each fraction carried down with it a certain amount of calcium which was not removed by dialysis, albumin holding about twice as much as globulin. Bendien and Snapper (38), however, from a study of the distribution of calcium between serum and *in vitro* ultrafiltrates containing various amounts of protein, concluded that calcium combined only with albumin. The present study indicates that *in vivo*, at least, both albumin and globulin combine with calcium in such a manner as to prevent its diffusion through pleural and peritoneal membranes.

If calcium combines with protein to form calcium proteinate, as Loeb believes, the relative abilities of albumin and globulin to bind calcium should be directly proportional to their general base-binding powers. At a pH of 7.35 Van Slyke, Hastings, Hiller, and Sendroy (39), by titrating the proteins in the presence of bicarbonate, found that the base combined with serum proteins was defined by the equation,  $BPr = 0.273 A + 0.189 G$ , in which BPr represents milli-equivalents of base combined with protein. This means that albumin binds  $0.273/0.189 = 1.44$  times as much base as does an equal quantity of globulin. This agrees closely with the relative calcium-binding powers of the two fractions derived from the data here presented,  $0.639/0.435 = 1.47$ . This gives strong support to the theory that calcium forms non-diffusible salts with protein.<sup>4</sup>

#### SUMMARY

1. Serum and transudates, simultaneously collected thirty-two times from twenty-five patients, have been analyzed for calcium, inorganic phosphorus, total protein, albumin, and globulin.

2. The difference between the concentration of calcium in serum and transudate is directly proportional to the difference in the

<sup>4</sup> Gutman and Gutman (40) by the statistical treatment of concentrations of calcium, albumin, and globulin in serum, according to principles similar to those applied by Hastings, Murray, and Sendroy (13), Peters and Eiser-son (14), and the author (see above), have come to the conclusion that euglobulin, at least in certain conditions which are characterized by hyper-proteinemia, combines with no appreciable amounts of calcium. Whether this can be established by more direct methods or not, it has no bearing upon the present conclusions which apply to sera of relatively normal constitution.

concentration of protein. The relation is described by the equation,  $\Delta\text{Ca} = 0.561 \Delta\text{Pr}$ .

3. The parts played by albumin and globulin in binding calcium are described by the equation,  $\Delta\text{Ca} = 0.639 \Delta\text{A} + 0.435 \Delta\text{G}$ .

4. The relative calcium-combining powers of the two protein fractions  $0.639/0.435 = 1.47$  agree closely with their relative base-combining powers, 1.44, as measured by Van Slyke, Hastings, Hiller, and Sendroy.

5. A calcium-protein complex is mainly responsible for the different calcium contents of serum and transudates.

6. This complex is probably a simple chemical compound, since the relative calcium-combining and base-binding powers of the protein fractions are of the same magnitude.

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# IONIZED CALCIUM OF SERUM AND TRANSUDATES IN VIVO\*

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(Received for publication, September 20, 1937)

In the preceding paper (1) it has been demonstrated that the differences between the concentrations of calcium in sera and transudates are directly correlated with the differences in the concentrations of protein in the two media, and that the relative amounts of calcium bound in non-diffusible form by albumin and globulin are proportional to the base-combining powers of these protein fractions. While this study was in progress McLean and Hastings (2) employed the isolated frog's heart, which is peculiarly sensitive to calcium ions, to measure the concentration of calcium ions in biological media. As a result of their studies they concluded that the ionized calcium in the blood depended upon an equilibrium between protein and calcium that can be described by the mass law equation

$$\frac{[\text{Ca}^{++}] \times [\text{Pr}^-]}{[\text{CaPr}]} = K \quad (1)$$

or, in negative logarithmic form,

$$p[\text{Ca}^{++}] + p[\text{Pr}^-] - p[\text{CaPr}] = pK_{\text{CaPr}} \quad (2)$$

$pK_{\text{CaPr}}$  varies with pH and with temperature, but, at 25° and pH 7.35, was found to be  $2.22 \pm 0.07$  for normal human serum, 2.24

\* This article represents work done in fulfillment of the thesis requirement for the degree of Doctor of Medicine at Yale University School of Medicine.

$\pm 0.08$  for purified serum globulin (beef and horse), and  $2.03 \pm 0.11$  for purified serum albumin.<sup>1</sup>

On both theoretical grounds and from certain observations McLean and Hastings were led to believe, also, that the calcium in serum ordinarily consists almost entirely of two fractions,  $\text{Ca}^{++}$  and  $\text{CaPr}$ , and that the ionized calcium is, within practical limits, identical with the diffusible fraction.

It is possible to prove that an equation of the type,  $\text{total Ca} = m\text{Pr} + b$ , which has been applied empirically to estimate Ca combined with protein from statistical data, by Hastings, Murray, and Sendroy (3), Peters and Eiserson (4), and the author (1), is only a special case in which  $[\text{Ca}^{++}]$  is kept constant by physiological regulation. Using data from the literature in which values for calcium and protein were given from subjects without disorders of calcification, and assuming that  $b$  in this equation represented the mean concentration of  $\text{Ca}^{++}$ , McLean and Hastings estimated that the mean  $\text{pK}_{\text{CaPr}}$  of human serum was 2.12. By similar treatment of the 331 routine observations mentioned in the preceding paper (1), the author has found  $\text{pK}_{\text{CaPr}}$  to be 2.10. These figures, if the assumptions on which their derivation depends are valid, have peculiar significance because they represent conditions existing in the circulating blood during life.

From comparisons of ultrafiltrates and dialysates culled from the literature, with the assumption that diffusible calcium is almost or entirely ionized, McLean and Hastings secured a value for  $\text{pK}_{\text{CaPr}}$  of 2.02, somewhat lower than that determined by the frog heart method. With the exception of Greene and Power's (5) vividiffusion experiments on dogs, the data utilized by McLean and Hastings were derived entirely from *in vitro* experiments.

From the simultaneous analyses of sera and transudates reported in the preceding article (1), with the same assumption that ionized and diffusible calcium are identical, it is possible to calculate  $\text{pK}_{\text{CaPr}}$  for each individual observation. For the purposes of these calculations it is necessary to convert concentrations of

<sup>1</sup> The same symbols will be used in this paper as in the last, protein = Pr, albumin = A, globulin = G. In addition ionization will be indicated in the conventional manner, and molar concentrations by brackets; *e.g.*;  $[\text{Ca}^{++}]$  = mm of calcium ions per kilo of water. Serum and transudates will be identified by the subscripts *s* and *f* respectively.

protein and calcium from the usual volumetric terms into terms of combining equivalents per kilo of water in serum and transudate respectively. The amount of water in a unit volume of these solutions has been estimated by the formula,  $98.6 - 0.75 \text{ Pr}$ . This formula, derived from data of Eisenman in 1935, differs little from that derived from more extensive data in 1936 by Eisenman, Mackenzie, and Peters (6). To convert gm. of protein to combining equivalents the factors of Van Slyke, Hastings, Hiller, and Sendroy (7) have been used, albumin and globulin being treated separately ( $\text{BPr} = 0.273 \text{ A} + 0.189 \text{ G}$ ). The values thus secured have been divided by 2 to convert milli-equivalents to mm, since McLean and Hastings have shown that protein acts as a divalent ion when it combines with calcium.

The effect of the Donnan equilibrium is neglected in these calculations, since the magnitude of the Donnan effect is quite uncertain. It is, then, assumed that  $[\text{Ca}^{++}]_s = [\text{Ca}^{++}]_f$ . In reality  $[\text{Ca}^{++}]_s = r[\text{Ca}^{++}]_f$ ,  $r$  being a constant representing the magnitude of the Donnan effect. Since the transudate is presumably in equilibrium with the serum in each individual case, the ionization constant  $K_{\text{CaPr}}$ , should be the same in both media. It is assumed that

$$[\text{Ca}] - [\text{Ca}^{++}] = [\text{CaPr}] \quad (3)$$

in both serum and transudate.

$$[\text{Pr}] - [\text{CaPr}] = [\text{Pr}^-] \quad (4)$$

also holds for both media.

Since  $K_{\text{CaPr}_s} = K_{\text{CaPr}_f}$  and  $[\text{Ca}^{++}]_s = [\text{Ca}^{++}]_f$  by substitutions and combinations of Equations 1, 3, and 4, it can be shown that

$$\frac{[\text{Pr}]_s - [\text{Ca}]_s + [\text{Ca}^{++}]}{[\text{Ca}]_s - [\text{Ca}^{++}]} = \frac{[\text{Pr}]_f - [\text{Ca}]_f + [\text{Ca}^{++}]}{[\text{Ca}]_f - [\text{Ca}^{++}]}$$

This equation can be solved readily for  $[\text{Ca}^{++}]$ , and  $\text{p}K_{\text{CaPr}}$  can then be calculated by means of Equation 2.

The data from which the calculations were made appear in Table I of the preceding paper. For reasons mentioned in that paper Observations 1, 6, 12, 23, and 30 were omitted. The results of the calculations appear in Table I.  $\text{p}K_{\text{CaPr}}$  varies from 1.87 to 2.50, with a mean of 2.07.



Observation 17 is particularly interesting because of the high value of  $pK$ , 2.50. In this case the concentration of inorganic phosphorus in serum and fluid was above 22 mg. per cent. In the

TABLE I  
*Values of  $pK_{CaPr}$*

Observation No.	$pK_{CaPr}$
2	1.93
3	1.93
4	2.24
5	2.21
5, <i>a</i>	2.28
5, <i>b</i>	2.10
7	1.99
8	2.33
9	1.96
10	2.03
11	2.07
13	2.04
14	1.87
15	1.89
16	2.05
(17)	(2.50)
18	1.96
19	2.22
20	2.03
21	2.20
22	2.01
24	1.94
25	2.07
26	1.93
27	1.88
28	2.09
29	2.13
Average.....	2.053
Standard deviation.....	0.127

light of the discovery by Smith (8) and Greenberg, Larson, and Tufts (9), that calcium may form a colloidal, non-diffusible compound with phosphate when the concentration of the latter is greatly increased, the high value of  $pK$  in this case may be false,

since part of the Ca not combined with protein may be combined in non-diffusible form with phosphate. With the exception of this case, however, no satisfactory correlation can be found between pK and inorganic phosphorus. When pK is plotted against the concentration of phosphate of serum, as it is in Fig. 1, there appears to be a rough correlation; but there are too few observations with high phosphate to permit any deductions concerning the quantitative effect of inorganic phosphorus upon the relation of calcium to protein.

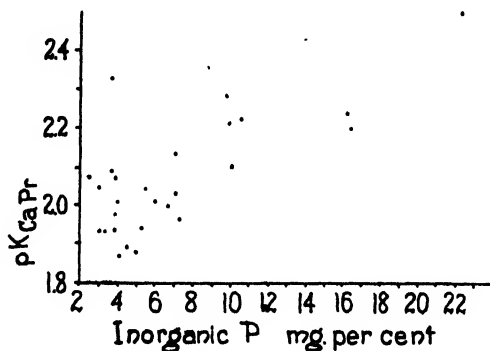


FIG. 1. The relation of inorganic phosphorus of serum to the estimated pK of calcium proteinate.

If Observation 17 is omitted, the mean value of  $pK_1$  is  $2.05 \pm 0.13$ . This confirms the observation of McLean and Hastings that diffusion methods yield lower values than the frog heart method. Whether this means that there is a small quantity of non-ionized diffusible calcium remains to be discovered.<sup>2</sup>

#### SUMMARY

By the application to analyses of human serum and transudates, simultaneously withdrawn, of the mass law equation derived by McLean and Hastings (2) relating calcium to protein, the value of  $pK_{CaPr}$  was found to be  $2.05 \pm 0.13$ .

<sup>2</sup> The discrepancy cannot be attributed either to failure to correct for the Donnan effect, or to the different temperatures at which the observations were made, because corrections for Donnan effect and temperature would only tend to reduce the value of pK.

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## NOTES ON THE EXTRACTION AND SAPONIFICATION OF LIPIDS FROM BLOOD AND BLOOD SERUM

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- Recently Boyd (5, 6) and Stewart and Hendry (19, 20) have published data on the extraction and saponification of lipids from whole blood, serum, and red blood cells. The following experiments were performed to test the modifications of the Stoddard and Drury titrimetric method for the determination of fatty acids (12), the gravimetric procedure for cholesterol (14), and for the colorimetric estimation of lipid phosphorus (11, 14) which have been used by us.

### EXPERIMENTAL

Ten experiments in each of which two samples have been hydrolyzed with sodium hydroxide and two with potassium hydroxide have been performed. Blood serum was used in seven of the ten experiments; in two a commercial lecithin was employed; in one the lipid material was a lecithin preparation from the liver of a guinea pig which had just been killed.

In Table I are the results of eighteen experiments in which six different samples of blood serum and twelve of whole blood were extracted by different methods and were analyzed for titrated fatty acids and lipid phosphorus. Cholesterol determinations were also made, but the values are omitted from Table I because the variations were not significant. All extractions were made in duplicate and the average of the two results is given in Table I. Before the measurement of each 2 cc. aliquot the blood was mixed to insure even distribution of cells and plasma. All the aliquots of blood were pipetted as rapidly as possible and were in alcohol and ether within 20 minutes from the measurement of the first to the measurement of the last sample. The alcohol and ether

TABLE I  
*Serum and Blood Lipid Values after Extraction by Different Methods*

Experiment No.	Titrated fatty acids					Lipoid phosphorus				
	Man and Gildea (1)	CO <sub>2</sub> extraction (2)	Boyd water hemolysis (3)	Blood water cooling (4)	Blood air cooling (5)	Man and Gildea (6)	CO <sub>2</sub> extraction (7)	Boyd water hemolysis (8)	Blood water cooling (9)	Blood air cooling (10)
1	m.-eq. 12.85	m.-eq. 12.55	m.-eq.	m.-eq. 12.77	m.-eq.	m.g. per 100 cc. 10.41	m.g. per 100 cc. 10.41	m.g. per 100 cc.	m.g. per 100 cc. 10.41	m.g. per 100 cc.
2	27.53			26.97		25.10			25.72	
3		11.67		10.73			8.01		7.90	
4	9.39	9.83		9.28			8.21		7.82	
5	11.13			10.51		8.56			8.34	
6	11.10	11.25		11.26		10.12	10.16		10.10	
Deviation from Man and Gildea										
Minimum . . . . .		-0.30		-0.94*			0		-0.39	
Maximum . . . . .		+0.44		+0.16*			+0.04		+0.62	
Average . . . . .		+0.10		-0.36*			+0.02		+0.07	



used in all extractions was a mixture of 3 parts of alcohol, redistilled after being refluxed with potassium hydroxide as detailed by MacArdle (10), and 1 part of ether, redistilled with an eight bulb pear column as described by King (9). These methods of distillation are the same as previously described by Man and Gildea (12). In every instance the ether was distilled within 28 hours of the time when it was used. The fat-free filter paper, either Schleicher and Schüll No. 589 or Whatman No. 43 (12), was reextracted for 4 to 6 hours with boiling redistilled alcohol. In all extraction methods the lipid extract was not diluted to volume until after filtration, washing of the filter paper and precipitate, and cooling of the extract to room temperature.

In Column 1 of Table I are given the results of the Man and Gildea extraction method which has been detailed previously (12). This consisted of the slow addition with shaking of 2 cc. of blood or serum to 25 cc. of alcohol-ether. This mixture was then refluxed for 1 hour at the lowest heat on a hot-plate insulated with four wire gauzes. The temperature in the refluxing flask rises slowly during the process of refluxing, but in one test experiment 90 minutes after the beginning of extraction the temperature registered 66° on a thermometer the bulb of which was in the boiling mixture. After 60 minutes of refluxing the lipid extract was filtered into a 50 cc. volumetric flask and the protein precipitate and filter paper were then washed with five to seven small portions of cold alcohol-ether until the volume of the extract was almost 50 cc.

In Column 2 of Table I are the results of the "CO<sub>2</sub> extraction" method which was identical to the process in the Man and Gildea extraction except that during the hour of refluxing a stream of carbon dioxide was admitted through the cork in the top of the Erlenmeyer flask. Carbon dioxide was added to prevent oxidation of fatty acids and an increase in carboxyl groups, after the suggestion of Stewart and Hendry (19).

In Table I, Column 5, is one experiment in which nitrogen was substituted for carbon dioxide during the hour of refluxing. In that same column is another single experiment called "6 hours heating in air." 2 cc. of blood were precipitated in 50 cc. of alcohol-ether. The alcohol-ether was then refluxed for 6 hours, a prolongation of the Man and Gildea technique without the use of either nitrogen or carbon dioxide.

The "Boyd water hemolysis" extraction followed the method for extraction of red blood cells, recommended by this author in August, 1936 (6). 2 cc. of blood were hemolyzed with 2 cc. of distilled water and 50 to 60 cc. of alcohol and ether were then added with constant agitation. The flasks were then shaken for 3 minutes before filtration. The precipitate and filter paper were washed with seven or more portions of alcohol and ether although Dr. Boyd used only one washing in some of his experiments ((6) p. 39).

The column labeled "Boyd, May, 1936" refers to the extraction methods recommended by Dr. Boyd at this time (5). For serum or plasma, in order to extract all the lipids, he adopted a dilution in which 3 cc. of serum or plasma were dropped slowly with shaking into 80 cc. of alcohol-ether. For blood he advised a dilution of 1:30 or 1:35. In all experiments on blood 2 cc. were precipitated in 75 cc. of alcohol-ether. Boyd stipulated that the extracts be heated for 5 minutes with frequent shaking to prevent superheating and after cooling, filtration, and washing, that the final volume should be 100 cc.

In the Bloor extraction 2 cc. of blood were added drop by drop with shaking to 26 cc. of alcohol-ether. The flasks were then immersed in boiling water and were shaken continuously to prevent superheating. After the alcohol-ether mixture appeared to boil, the flasks were kept immersed in the hot water for 1 minute. In Column 4 of Table I called "Bloor water cooling" are given the results on the lipid extracts when the containing flasks immediately after removal from the hot water were cooled in cold water and the extracts were filtered immediately after cooling (1, 2). In contrast, Column 5 called "Bloor air cooling" contains the results on the extracts when the flasks after immersion in hot water were not chilled in cold water but were allowed to cool spontaneously to room temperature (3, 4). This air cooling required 40 to 60 minutes, a length of time almost as great as in the Man and Gildea refluxing method. In both of these Bloor methods the extract was filtered into a volumetric flask, the precipitate and filter paper were washed five to seven times, and the extract was made up to volume subsequent to the processes of filtration and washing.

The method of extraction devised by Man and Gildea was designed to be applicable to both serum and whole blood, so that



when lipids were to be analyzed simultaneously in both serum and whole blood every step in the technique would be comparable. Since it is generally assumed that whole blood is more difficult to extract than serum or plasma, only six experiments on serum, but twelve on whole blood, were conducted to reinvestigate extraction methods.

At the end of the six experiments on serum and the twelve experiments on blood are given the average deviations of the yields of each extraction method compared with the yields of the Man and Gildea technique. A negative sign indicates that the yield was lower than by the Man and Gildea method. Average deviations have been calculated after consideration of positive and negative signs. This means that, if the differences in yields by different extraction methods were only governed by the laws of chance, variations should cancel each other and the average deviation would approach zero. The minimum and maximum values represent the extreme differences from the Man and Gildea yields. In comparing the yields of lipids obtained by different methods one criterion of a significant variation is that obtained by a statistical analysis of the duplicates of 100 consecutive determinations of serum lipids. It was found that, given the average of a pair of duplicate fatty acid determinations, the average of two other duplicates should agree within  $\pm 0.36$  milli-equivalent of fatty acid, or given one duplicate, the other can be estimated within  $\pm 0.49$  milli-equivalent. Given the average of a pair of duplicate lipid phosphorus determinations, the average of another pair of duplicates should agree within  $\pm 0.21$  mg. per cent of lipid phosphorus.

### *Results*

#### *Sodium Hydroxide Compared with Potassium Hydroxide in Saponification*

In the saponification experiments there was much overlapping of the four fatty acid values obtained after hydrolysis of two samples with sodium and two with potassium hydroxide. This overlapping prohibits drawing any distinction between these two saponifying agents. Although in seven of the ten experiments the average of the two potassium hydroxide duplicates was higher than the average of the two sodium hydroxide duplicates, in three sodium hydroxide gave higher yields of fatty acids than potassium

hydroxide. With the exception of the crude lecithin all the lipids saponified in these experiments were not dried or exposed to the oxidizing influence of air between extraction or purification and saponification. It is not known whether the differences between these two saponifying agents may be minimized when the phosphatides are saponified immediately after extraction or purification.

#### *Effect of Refluxing on Completeness of Extraction*

The data in Table I show that for whole blood refluxing for 1 hour increases the yield of fatty acids by alcohol and ether extraction. This increase is apparent in samples which have been protected from oxidation by carbon dioxide and therefore is not dependent on decomposition of the fats or upon an increase in the number of carboxyl groups. Because the differences between the serum fatty acid values obtained by the Man and Gildea and the Bloor water cooling methods are not uniform, it would seem that some sera are more difficult to extract than others and that the hour of refluxing is advisable for those sera which are difficult to extract.

Usually there is a greater yield of lipid phosphorus after refluxing blood for 1 hour with alcohol and ether than after heating the alcohol, ether, and blood mixture for 1 to 5 minutes. Comparison of the values for lipid phosphorus in the extracts which have been refluxed in air and carbon dioxide shows that in nine of the eleven experiments on whole blood higher yields of lipid phosphorus were obtained in those samples which were refluxed in the presence of carbon dioxide. In the two experiments on blood serum the differences were less than 0.05 mg. per cent. In the one experiment, when whole blood was refluxed for 1 hour in air, in the presence of carbon dioxide and of nitrogen the three values agreed within 0.14 mg. per cent. The above results suggest that, when the somewhat unstable phosphatides in whole blood are protected by an inert gas, higher quantities of phospholipid may be obtained. Since the method of refluxing in air gave higher results than those without refluxing, the yields in the carbon dioxide-refluxed samples are appreciably larger than in the non-refluxed samples.

Statistical analysis of 100 consecutive samples of serum cholesterol showed that, given the average of two duplicates, the average of another pair can be estimated within a probable error

of  $\pm 7.69$  mg. per cent. It has been concluded that any of the above methods is equally good for the extraction of total cholesterol, because in a comparison of cholesterol yields of all different methods the mean value, regardless of sign, of the 98 differences was only 5.96 mg. per cent. Page, Kirk, Lewis, Thompson, and Van Slyke in two experiments on plasma found no significant differences between the amounts of total and free cholesterol in extracts made by the Bloor and the Man refluxing methods (18).

#### DISCUSSION

The saponification experiments showed no significant difference between hydrolysis with potassium and sodium hydroxide. These results do not substantiate the saponification experiments originally published by Man and Gildea (12). Stewart and Hendry (19) have very justly criticized these original findings of ours.

In our original calculations (12) it was concluded that only 82 per cent of the fatty acids of phosphatides were saponified by our method, because we had not been able to recover more than approximately 80 per cent of the theoretical fatty acids saponified from a purified sample of lecithin. The proportion of fatty acids to be ascribed to phosphatides in blood or serum still remains obscure, because these phosphatides are not homogeneous but are composed of a variety of compounds. In view of this uncertainty it seems better to us not to attempt to correct titrated fatty acids for phosphatide fatty acids as we did previously or to try to calculate non-phosphatide fatty acids. Now the fatty acids are calculated directly from the titer and are called "Titrated fatty acids." The last sentence before the summary in our original paper has, therefore, no significance. "The value obtained in Equation 1 + 18.0 per cent phospholipid fatty acids is considered to be equivalent to the total fatty acid content of the serum, as far as can be judged at the present time" (12). This step in the calculations has affected the fatty acid values by about 10 per cent or less in a number of our published papers. Fortunately, the omission of this step in calculation does not alter the relative values or conclusions reported in previous studies.<sup>1</sup>

<sup>1</sup> To change to "Titrated fatty acids" "Total fatty acids" in the following papers already published (7, 8, 13, 14, 17) multiply the lipoid phosphorus by 2/31 and subtract 18 per cent of this value from the "Total fatty acids."

It has been shown above that higher yields of fatty acids and of phosphatides are obtained from whole blood and to a lesser degree from serum when the alcohol-ether mixture is refluxed for 1 hour than when the Bloor or the Boyd red blood cell extraction method is used.

Boyd (5) questioned the refluxing modification because it required more time and apparatus. In his method the extracts were heated 5 minutes and, "When cool, the extracts were filtered" ((5) p. 225). It was found in our laboratory that 40 to 60 minutes were necessary for the extracts to reach room temperature when they were allowed to cool spontaneously. This air cooling method was the one apparently recommended by Bloor in 1922 and 1928 (3, 4) and must be contrasted with the quicker water cooling methods of 1914 and 1915 (1, 2). There is no appreciable difference in time between refluxing the alcohol-ether mixture for 1 hour as recommended by Man and Gildea and between heating the extract for 5 minutes and then letting it cool 40 to 60 minutes before filtration.

While it is true that the refluxing of the alcohol-ether mixture does require condensers and some heating device, this initial expense for apparatus is offset in Boyd's modification by the expense of large amounts of solvent. To obtain the greatest extraction of lipids from serum when the samples were not refluxed he recommended the use of at least twice as much solvent (5) as Man and Gildea had found necessary. He has discussed in detail the dilutions recommended by Bloor at different times. In addition to the expense of these quantities of alcohol and ether the large volume in which the lipids are subsequently dissolved has its disadvantages. More time is required for the removal of the solvent from the lipids. In the case of the phosphatides which have been shown to be unstable and easily broken down (11) the additional heating and time for removal of the solvents may easily result in decomposition and a marked loss of phospholipids.

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To change "Non-phospholipid fatty acids" in the following papers (13, 15-17) to "Titrated fatty acids" multiply the lipoid phosphorus by  $2/31$  and add 82 per cent of this value to the non-phospholipid fatty acids. In these calculations it was assumed that one combining weight of phosphorus, 31, unites with 2 of fatty acid in each gm. molecule of serum phosphatide, and that only 82 per cent of the phosphatide fatty acids was actually saponified and titrated.

In his dilution experiments on serum he found that, "Phospholipid and free cholesterol were extracted almost as readily with the smaller volumes as with the larger, but with smaller volumes the extraction of neutral fat and cholesterol esters was much more incomplete" ((5) p. 225). But in the experiments on whole blood he stated that, "The phospholipid and free cholesterol only of these extracts were analyzed" ((5) p. 226). Since these two fat fractions were more easily extractable from serum, their use as criteria for the efficiency of extraction from whole blood can be criticized.

Boyd (5, 6) and Stewart and Hendry (19) have questioned in the Man and Gildea modification of the Stoddard and Drury titrimetric method the refluxing process, because it may cause an increase in titratable substances in the extract. Stewart and Hendry found in samples of blood refluxed 5 hours, five times as long as the samples are refluxed in the Man and Gildea modification, that there was an increase of 40 per cent in the titer of fatty acids. They do not state the temperature of their heating apparatus. They found that if the blood was refluxed in a "continuous current of purified hydrogen" this increase in fatty acids did not occur. For this reason in fifteen of our eighteen experiments refluxing was conducted in the presence of air and also in carbon dioxide. In seven of the fifteen experiments higher titers were obtained in the carbon dioxide samples and in eight experiments higher yields in the air specimens. In one experiment in which nitrogen was used instead of carbon dioxide and in another single experiment, when the alcohol-ether blood mixture was refluxed for 6 hours, the fatty acids all agreed within 0.43 milli-equivalent. These results show that the high yields of whole blood fatty acid obtained after refluxing for 1 hour, as recommended by Man and Gildea, represent a greater extraction of fatty acids and are not caused by oxidative decomposition of the fatty acid chains and an augmentation of carboxyl groups.

Boyd (6) has presented evidence to show that heating alcohol-ether extracts of red blood cells extracts colored material which contaminates the lipid material. "The colored material was found to be soluble in alcohol, ether, acetone, petroleum ether, methyl alcohol, chloroform, and dilute sodium hydroxide solution; it was insoluble in water and dilute hydrochloric or sulfuric acid" (p. 41).

The fact that this substance is soluble in these lipid solvents makes it difficult to judge whether such a colored substance should be classed as a contaminant of lipids or as a lipid itself. However, the differences in Table I of 0.01 to 2.65 milli-equivalents in the fatty acids of whole blood by the Man and Gildea refluxing process and the Boyd hemolysis extraction method without heat do not approach the differences in fatty acid found by Boyd in extracts free from or contaminated with this colored substance. He found the neutral fat three times as high after 1 hour's heating as without heat ((5) p. 228). Boyd states that, "When the colored material was dissolved in 0.1 N NaOH and this extracted with petroleum ether, the color remained in the aqueous alkaline medium and did not pass visibly into the petroleum ether" ((6) p. 41). This insolubility in petroleum ether is no proof that the "contaminating substance" is non-lipid in nature, because petroleum ether does not extract quantitatively all lipids from an aqueous alkaline solution. Boyd himself has presented evidence to show that extraction of red blood cells without heating gave values for total fatty acids below 240 mg. per cent, while there were 275 mg. per cent of fatty acids in the samples which had been both heated 15 to 60 minutes and treated for the removal of the colored "contaminating substance" ((6) Fig. 2). If a short period of heating can result in 13 per cent higher yields of fatty acid, this increase in determined fatty acids after 60 minutes of heating cannot be attributed entirely to contaminating substances. With the present definition of lipids, it seems to the authors that the additional titration value for fatty acids in the Man and Gildea refluxed samples above that in samples not heated or heated for a short time may represent a more complete extraction of lipid material from whole blood.

#### SUMMARY

In ten experiments in which lipid extracts were saponified simultaneously with sodium and potassium hydroxides no significant differences in fatty acids were found.

Eighteen experiments were conducted in which serum or blood was extracted by the Bloor method, by the Boyd modifications, and by refluxing for 1 hour in the presence of air and carbon dioxide. Data have been presented to prove that the Man and

Gildea refluxing process extracts more material which by present definition is classed as lipid in nature than the Boyd modifications of the Bloor extraction method.

When whole blood was used, the refluxing process gave yields of fatty acid 0.01 to 2.65 milli-equivalents higher than did the other methods except for one analysis. Serum gave slightly larger amounts of fatty acid after the refluxing process, the average difference being 0.36 milli-equivalent. Samples refluxed in air and in carbon dioxide yielded identical amounts of fatty acids.

Phosphatides of whole blood were highest in the aliquots refluxed for 1 hour in the presence of carbon dioxide and exceeded the samples refluxed in air by an average deviation of 0.26 mg. per cent of lipid phosphorus. The air refluxing method gave higher yields than the other extraction methods by average variations of 0.09 to 0.41 mg. per cent of lipid phosphorus.

Cholesterol determinations in all extracts agreed satisfactorily.

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## SPECIFICITY OF INTESTINAL AMINOPOLYPEPTIDASE\*

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In a previous communication (1) it was noted that intestinal aminopolypeptidase, prepared according to Waldschmidt-Leitz and Balls (2), attacked sarcosyldiglycine, a substrate lacking an intact amino group. In all preparations studied, the sarcosyldiglycine activity was proportional to the triglycine (aminopolypeptidase) activity. Aminopolypeptidase is generally assumed to require a free amino group. In their specificity studies of this enzyme, Waldschmidt-Leitz and Balls (2) demonstrated the essential nature of a basic nitrogen atom, but did not specifically show that an intact amino group was necessary. If only a basic nitrogen atom is necessary, prolyl peptides should also be hydrolyzed by aminopolypeptidase. Balls and Köhler (3) prepared a purified aminopolypeptidase which they considered, on the basis of good evidence, to be enzymatically pure. They tested, however, neither prolyl peptides nor N-methyl peptides. Investigations from the laboratories of Grassmann (4) and Abderhalden (5) have led to the conclusion that since prolinase activity of various preparations did not parallel either aminopolypeptidase or dipeptidase activity, neither of these enzymes was responsible for the hydrolysis of prolyl peptides. In these investigations, however, leucyldiglycine was employed to determine aminopolypeptidase activity, and leucylglycine to determine that of dipeptidase. These peptides have since been shown (1) to measure primarily leucyl peptidase rather than aminopolypeptidase activity; therefore, the conclusions regarding prolinase lose much of their validity.

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*Specificity of Aminopolypeptidase*—Because of these ambiguities in existing knowledge of aminopolypeptidase specificity, it was thought desirable to investigate the behavior of intestinal aminopolypeptidase toward substrates lacking an intact amino group. Table I shows the activity of a typical aminopolypeptidase preparation toward a number of substrates. It will be noted that the N-methyl tripeptides are attacked, but more slowly than the corresponding unsubstituted peptides. The dimethyl-substituted

TABLE I  
*Specificity of Ereptic Aminopolypeptidase*

Substrate	Enzyme per 3 cc. of reaction mixture	Incubation time	Titration increase for 1 cc. of reaction mixture	Hydrolysis	Approximate relative hydrolyzability
	cc.	hrs.	cc. N/15	per cent*	
<i>dl</i> -Alanyldiglycine . . . . .	0.1	1	0.238	48	1000
Triglycine . . . . .	0.2	1	0.292	58	600
<i>dl</i> -Leucyldiglycine . . . . .	1.0	1	0.250	50	100
<i>dl</i> -N-Methylalanyldiglycine . . . .	0.5	1	0.330	66	270
Sarcosyldiglycine . . . . .	1.0	1	0.266	53	110
<i>dl</i> -N-Methylleucyldiglycine . . . .	1.0	3	0.022	4	2
“ . . . . .	1.0	8	0.042	8	2
<i>dl</i> -N-Dimethylalanyldiglycine . . .	1.0	2	-0.006	-1	
“ . . . . .	1.0	19	0.000	0	0
<i>dl</i> -Prolyldiglycine . . . . .	0.2	1	0.346	69	700
<i>dl</i> -Alanylglycylmethylamine . . . .	1.0	2	0.166	33	35
<i>dl</i> -Prolylglycine . . . . .	1.0	2	0.104	21	20
<i>dl</i> -Alanylglycine . . . . .	1.0	2	0.250	50	50
Diglycine . . . . .	1.0	3	0.136	27	20
<i>dl</i> -Leucylglycine . . . . .	1.0	3	0.072	14	10

\* Per cent of one linkage (racemic peptides, per cent of one linkage of one optical component).

peptide N-dimethylalanyldiglycine is not hydrolyzed, indicating that at least 1 hydrogen atom must be present on the basic nitrogen atom, possibly because the dimethylated peptide is unable, because of steric hindrance, to combine with the enzyme. Removal of the carboxyl group from alanyldiglycine greatly decreases its rate of hydrolysis, but the fact that alanylglycylmethylamine is hydrolyzed supports the conclusion of Waldschmidt-Leitz and Balls (2) that the carboxyl group is not essential. Prolyldiglycine

is split very rapidly, alanyldiglycine being the only substrate found which exceeds it in rate of hydrolysis.

The dipeptides prolylglycine, alanylglycine, glycyglycine, and leucylglycine are slowly split. Waldschmidt-Leitz and Balls (2) reported the preparation of ereptic aminopolypeptidase entirely free from leucylglycine activity, but we have never been able to obtain any such preparations, even with intestinal mucosa from their laboratory, and by the use of their preparative and analytical methods. In fact, the relative constancy of the residual "dipeptidase" activity of all aminopolypeptidase preparations seemed to be an indication that aminopolypeptidase itself was the enzyme responsible for the slow dipeptide hydrolysis. The aminopolypeptidase preparations of Tables III and IV of the preceding paper (1) had very much the same relative specificity toward various peptides as the preparation of Table I above. At the time of the previous work, however, no particular significance was attached to the slow dipeptide hydrolysis, since it was then considered to be due to the presence of traces of dipeptidase.

*Enzymatic Purity of Aminopolypeptidase*—In order to determine whether all the types of substrates shown in Table I were being hydrolyzed by the same enzyme, an aminopolypeptidase solution was treated in various ways, and after each treatment its activity was determined on five different substrates. The results obtained are summarized in Table II. The data indicate strongly that all the substrates employed, with the possible exception of alanylglycine, are hydrolyzed by the same enzyme. Acetone-precipitated aminopolypeptidase, which, according to Balls and Köhler (3) is enzymatically pure, splits the substrates at the same relative rates as before precipitation. Adsorption and elution also leave the specificity without significant change, except that the eluates are somewhat low in relative alanylglycine activity. It is apparent that prolyldiglycine, triglycine, N-methylalanyldiglycine, and prolylglycine are all split by the same enzyme. It will be noted that the relative activity toward triglycine and N-methylalanyldiglycine is lower in crude erepsin than in aminopolypeptidase. This may indicate the presence in crude erepsin of a prolyldiglycine-splitting enzyme other than aminopolypeptidase. It should be pointed out, however, that these two substrates have a much lower enzyme affinity than prolyldiglycine,

and are more subject to competition from inhibitory substances in crude erepsin.

It is clearly shown in Table II that crude erepsin contains a prolylglycine-splitting enzyme distinct from aminopolypeptidase. If the conclusion of Bergmann and coworkers (6) that dipeptidase requires an intact amino group is valid, this enzyme cannot be

TABLE II  
*Effect of Fractionation on Aminopolypeptidase Specificity*

Enzyme Preparation No.*	Prolyldiglycine activity†	Relative activity toward other substrates (prolyldiglycine activity = 1)			
		Triglycine	N-Methylalanyl- anyldiglycine	Prolylglycine	Alanylglycine
	<i>units per l.</i>				
I	30	0.6	0.2	0.12	1.8
II	16.9	0.9	0.5	0.025	0.07
III	10.8	0.9	0.5	0.025	0.08
IV	11.5	0.7	0.4	0.025	0.08
V	3.7	0.8	0.5	0.024	0.05
VI	4.1	0.8	0.4	0.027	0.05
VII	9.7	0.8	0.5	0.028	0.07
VIII	5.5	0.8	0.5	0.027	0.06

\* Preparation I is acetic acid erepsin (1); Preparation II, aminopolypeptidase; Preparation III, Preparation II precipitated with 1.2 volumes of acetone (3); Preparation IV, 15 cc. of Preparation II, after adsorption at pH 5.3 with 13 mg. of  $\beta$ -Fe<sub>2</sub>O<sub>3</sub>·H<sub>2</sub>O; Preparation V, the adsorbate from Preparation IV eluted with 5 cc. of 0.0025 M Na<sub>2</sub>HPO<sub>4</sub>; Preparation VI, another preparation similar to Preparation V; Preparation VII, 10 cc. of Preparation II, after adsorption at pH 6.5 with alumina C<sub>γ</sub> (6 mg. of Al<sub>2</sub>O<sub>3</sub>); Preparation VIII, the adsorbate from Preparation VII eluted with 5 cc. of 0.005 M Na<sub>2</sub>HPO<sub>4</sub>.

† Corrected for volume changes during manipulation; referred to glycerol extract of hog intestine.

dipeptidase. However, further investigation is necessary to determine the nature of this peptidase.

*Dipeptide Hydrolysis by Aminopolypeptidase*— Since the relative alanylglycine activity of the enzyme preparations of Table II shows some variation, and since other workers have concluded that aminopolypeptidase does not attack dipeptides, further experiments were carried out in order to determine whether the

TABLE III  
Effect of  $H_2S$  on Hydrolysis of Alanylglycine and Triglycine

Enzyme	Enzyme per 3 cc. of reaction mixture	Incu- bation time	Substrate	Hydroly- sis	Inhibi- tion
	cc.	hrs.		per cent	per cent
Acetic acid erepsin	0.05	1	Alanylglycine	49	
	0.05	1	" + $H_2S$	1	98
	0.2	1	Triglycine	71	
	0.2	1	" + $H_2S$	67	8
Aminopolypeptidase (similar to Prepara- tion II, Table II)	1.0	2	Alanylglycine	52	
	1.0	2	" + $H_2S$	29	44
	0.2	1	Triglycine	62	
	0.2	1	" + $H_2S$	52	24
Aminopolypeptidase (similar to Prepara- tion V, Table II)	1.5	2	Alanylglycine	30	
	1.5	2	" + $H_2S$	18	40
	0.3	1	Triglycine	47	
	0.3	1	" + $H_2S$	39	22

The  $H_2S$ -containing substrates contained 0.0025 M  $H_2S$ -NaHS buffer (pH 8). In calculating per cent inhibition, account was taken of the fact that triglycine hydrolysis is a first order reaction, while alanylglycine hydrolysis is a zero order reaction.

TABLE IV  
Inhibition of Alanylglycine Hydrolysis by Leucyldiglycine

Enzyme	Substrate	Hydrolysis after 1 hr.
		per cent*
Aminopolypeptidase	<i>dl</i> -Leucyldiglycine	47
"	<i>dl</i> -Alanylglycine	25
"	<i>dl</i> -Leucyldiglycine + <i>dl</i> -alanylglycine	46
Acetic acid erepsin†	<i>dl</i> -Leucyldiglycine	12
" " "	<i>dl</i> -Alanylglycine	50
" " "	<i>dl</i> -Leucyldiglycine + <i>dl</i> -alanylglycine	58

\* Per cent of one linkage of one substrate.

† 0.0033 M  $MgCl_2$  was present in the cases in which acetic acid erepsin was used.

alanylglycine activity of aminopolypeptidase preparations was due to traces of dipeptidase. Table III shows that the dipeptidase of crude erepsin is completely inhibited in the presence of

0.0025 M  $H_2S$ , while aminopolypeptidase is only partially inhibited. (The alanyl-glycine activity of crude erepsin is due almost entirely to dipeptidase, since this peptide is hydrolyzed only very slowly by leucyl peptidase and aminopolypeptidase.) The alanyl-glycine activity of aminopolypeptidase preparations, however, is not completely inhibited by  $H_2S$ , showing that dipeptide splitting is here not due to traces of dipeptidase, but presumably to the aminopolypeptidase itself.

Since leucyl-diglycine has a high affinity for aminopolypeptidase, the presence of this peptide should inhibit alanyl-glycine hydrolysis by aminopolypeptidase. In Table IV, data are given which show that such is in fact the case. Leucyl-diglycine inhibits alanyl-glycine splitting by aminopolypeptidase much more than it inhibits alanyl-glycine hydrolysis by the dipeptidase of crude erepsin. This is additional evidence that aminopolypeptidase splits dipeptides.

#### DISCUSSION

It is clear from the foregoing data that intestinal aminopolypeptidase does not require an intact amino group in its substrate. Waldschmidt-Leitz and Balls (2) concluded that, since this enzyme did not hydrolyze acylated peptides, the amino group was essential. It is, however, apparently more nearly correct to assume that the amino group may be substituted provided the substituent does not destroy the basic character of the nitrogen atom. Such an assumption is in accord with the present data, which show that methylated peptides and prolyl peptides are readily split. In fact, intestinal aminopolypeptidase splits prolyl-diglycine more rapidly than leucyl-diglycine or glycyldiglycine, and almost as rapidly as alanyl-diglycine. The whole of the prolyl-diglycine activity of crude erepsin may be ascribed to its aminopolypeptidase content; hence, the existence of a tripeptide-hydrolyzing prolinase remains to be demonstrated. However, only a small part of the prolyl-glycine activity of crude erepsin is due to aminopolypeptidase.

The present data also indicate that aminopolypeptidase slowly attacks certain dipeptides, although the possibility has perhaps not been absolutely excluded that the dipeptide splitting observed may be due to some contaminating peptidase.

*Methods*

In general, the experimental methods were those of a previous investigation (1). Degree of hydrolysis was determined by use of the Linderström-Lang titration (7). The maximum difference encountered between duplicate determinations was 0.01 cc. of  $N/15$  acid, corresponding to 2 per cent hydrolysis of the substrate. Hydrolysis of prolyldiglycine and alanylglycine by aminopolypeptidase was found to be a zero order reaction, while the splitting of triglycine, prolylglycine, and *N*-methylalanyldiglycine proceeded as a first order reaction. In calculating activity units, therefore, the linear formula previously described (1) was used for the first group of substrates, while the monomolecular formula was used for the second group. In all cases the substrate concentration in the hydrolysis mixture was  $M/30$  ( $M/15$  for racemic peptides). The following examples will make the method of calculation clear.

3 cc. of hydrolysis mixture, containing 0.2 cc. of enzyme, gave, after 60 minutes incubation, a titration increase per 1 cc. aliquot of 0.292 cc. of  $N/15$  HCl. Assuming the hydrolysis to be a first order (monomolecular) reaction, the formula  $E = \log(a/(a - x))/2t$  is used, where  $E$  is the number of enzyme units present in the reaction mixture,  $t$  the hydrolysis time in minutes (here 60),  $a$  the titration increase corresponding to 100 per cent hydrolysis (here 0.500), and  $x$  the observed titration increase (here 0.292). Therefore, there are present in the 0.2 cc. enzyme sample  $\log(0.500/0.208)/120 = 0.0032$  enzyme units. The factor 2 in the denominator of the above formula was introduced in order to make the present units directly comparable with those in earlier work (8), where 6 cc. of reaction mixture, and 2 cc. titration aliquots, were used. If, in the above example, the hydrolysis had been a zero order (linear) reaction, the formula  $E = 0.602x/t$  would have been used; the enzyme content of the sample in such a case would therefore have been 0.0029 unit. The arbitrary factor 0.602 in the above linear formula has been introduced in order that the two types of units may be as nearly as possible directly comparable. That is, half hydrolysis of the substrate ( $x = 0.250$ ) in 60 minutes corresponds to 0.0025 unit whichever formula is used.

The substrate solutions as used were half neutralized; that is, the solution was an equimolecular mixture of the peptide and its sodium salt. In the case of the decarboxylated peptide, alanyl-glycylmethylamine, the substrate was an equimolecular mixture of the free base and its acid salt. Substrates thus made were found in all cases to maintain the reaction mixture during hydrolysis between pH 8 and pH 8.3. Enzyme-substrate mixtures were incubated at 40°.

Acetic acid crepsin (1) was made by adding to glycerol extract of hog duodenal mucosa an equal volume of 0.06 N acetic acid, centrifuging off the precipitated impurities, and adjusting to pH 7. A typical preparation of aminopolypeptidase was made as follows: 150 cc. of acetic acid crepsin were adsorbed at pH 5 with nine portions of a suspension in 50 per cent glycerol of  $\beta\text{-Fe}_2\text{O}_3 \cdot \text{H}_2\text{O}$  (1). The total  $\text{Fe}_2\text{O}_3$  added was 4.8 gm. The solution was then adjusted to pH 7, dialyzed overnight to remove most of the glycerol, and concentrated under reduced pressure to a volume of 250 cc. Concentration was found desirable because of the volume increase during dialysis.

The alanyl, leucyl, and glycyl peptides were made by the methods of Fischer (9). The methylated peptides were made in an analogous manner, by the reaction of monomethylamine or dimethylamine instead of ammonia with the various halogenacyl compounds. The prolyl peptides were made by the procedure of Grassmann, von Schoenebeck, and Auerbach (4). The 2,5-dibromopentanoic acid used in the synthesis was made by the method of Merchant, Wickert, and Marvel (10). This method was found to give much better yields than the procedure used by Grassmann and others. The over-all yield from trimethylene bromide was 47 per cent. *dl*-Prolyldiglycine, nitrogen (Kjeldahl) 18.4 per cent, calculated 18.3 per cent; neutral equivalent (acetone-HCl (7)) 229, calculated 229. *dl*-Prolylglycine, nitrogen (Kjeldahl) 16.3 per cent, calculated 16.3 per cent; neutral equivalent (acetone-HCl) 173, calculated 172.

Alanylglycylmethylamine was prepared as follows: Glycylmethylamine hydrochloride was treated, in aqueous solution, in the presence of excess  $\text{K}_2\text{CO}_3$ , with  $\alpha$ -bromopropionyl chloride. The temperature was kept below 0°. The product crystallized out of the reaction mixture while the acid chloride was being

slowly added. Additional product was obtained from the mother liquor by chloroform extraction. The product was recrystallized from chloroform-toluene, and let stand 2 days at 25° with a large excess of 25 per cent aqueous ammonia. Upon concentration and addition of alcohol, the hydrobromide of the decarboxypeptide crystallized. The yield, based on glycylmethylamine, was 51 per cent of theory. Neutral equivalent (alcohol-NaOH, thymolphthalein) 241, calculated 240.

The glycylmethylamine was prepared by allowing a chloroform solution of chloroacetyl chloride to react, below 0°, with aqueous methylamine. The resulting N-methylchloroacetamide was converted to glycylmethylamine hydrochloride by 3 hours treatment at room temperature with excess aqueous ammonia. The yield, based on chloroacetyl chloride, was 70 per cent.

#### SUMMARY

1. The intestinal peptidase usually called aminopolypeptidase does not require an intact amino group. A basic nitrogen atom, carrying at least 1 hydrogen atom, appears to be necessary.

2. This enzyme therefore is readily able to hydrolyze prolyl peptides, previously thought to be attacked only by prolinase.

3. Intestinal aminopolypeptidase slowly attacks the dipeptides alanylglycine, leucylglycine, diglycine, and prolylglycine.

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## THE CHEMISTRY OF VITAMIN E TOCOPHEROLS FROM VARIOUS SOURCES\*

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In a previous communication (1) some of us described the isolation from wheat germ oil of an alcohol,  $C_{29}H_{50}O_2$ , having marked vitamin E activity, for which we proposed the name  $\alpha$ -tocopherol. The substance itself was an oil, and was isolated in the form of the allophanate melting at 158–160° from which it could be easily regenerated by alkaline hydrolysis. From it was also obtained a *p*-nitrophenylurethane, crystallizing in fine needles melting at 129–131°. The substance was believed to be homogeneous because adsorption of the allophanate on a column of calcium carbonate from benzene solution gave end-fractions of the same melting point, which on hydrolysis yielded alcohols of the same biological activity. Furthermore,  $\alpha$ -tocopherol from the allophanate was converted to the *p*-nitrophenylurethane, which was recrystallized to constant melting point, and then reconverted to the allophanate which, after a few recrystallizations, melted at 158–160°. The extra step in purification effected no change in the biological activity or in the absorption spectrum of the  $\alpha$ -tocopherol.

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Dr. T. R. Hogness and Dr. F. P. Zscheile, Jr., of the Chemistry Department, University of Chicago, very kindly measured the absorption spectrum of  $\alpha$ -tocopherol in isooctane. They found that although the shape of our curve was correct, the absolute

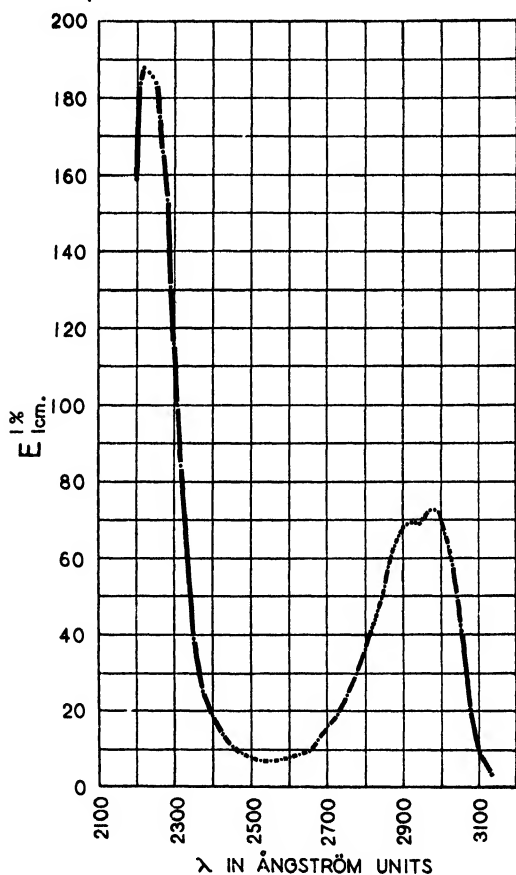


FIG. 1

values were too high, at the 2980 Å. maximum  $E_{1\text{cm.}}^1\% = 73$  instead of 90. Drummond and Hoover (2) reported 75. Since Hogness and Zscheile were able to extend their measurements into the much further ultraviolet, we are reproducing their curve (Fig. 1).

As mentioned in the previous communication, there was also isolated from wheat germ oil a beautifully crystalline allophanate melting at 136–138°, the analysis of which indicated that it was derived from an isomer of  $\alpha$ -tocopherol. In spite of its homogeneous appearance and the fact that this allophanate could be recrystallized a number of times from alcohol without change in melting point, it was possible by careful recrystallization from acetone to bring the melting point up to 144–146°. This allophanate appeared to have a slight optical rotation. The alcohol from this allophanate was very similar in its chemical properties and absorption spectrum to  $\alpha$ -tocopherol, but had distinctly less biological activity. For it the name  $\beta$ -tocopherol is proposed.

A short report (3) referred to the isolation of two tocopherols from cottonseed oil, one of which appeared to be identical with  $\alpha$ -tocopherol from wheat germ oil.

We now wish to report in more detail our work with cottonseed oil, and, in addition, the isolation of tocopherols from lettuce leaves and palm oil. Olcott and Mattill (4) prepared a potent concentrate of vitamin E from lettuce leaves, which was of particular interest in that it represented a source quite different from the seed oils previously investigated. Moreover, they reported that their concentrate showed merely general absorption of increasing intensity toward the shorter ultraviolet, without any definite maximum between 2900 and 3000 Å. (5). Palm oil possessed interest in that Olcott (6) reported a preparation of its non-saponifiable matter to possess strong absorption at 2940 Å. without corresponding biological activity.

From each of the three sources an allophanate has been secured which appeared to be identical with that of  $\alpha$ -tocopherol from wheat germ oil in that the preparations showed the same melting point, showed no depression on mixed melting, agreed reasonably well in analysis, and on hydrolysis yielded alcohols having, within the limits of accuracy of the test, the same degree of biological activity (Table I). Further, the preparations from cottonseed oil and lettuce were converted to the *p*-nitrophenylurethane, which gave no melting point depression when mixed with that of  $\alpha$ -tocopherol from wheat germ oil, or with each other.

From lettuce, only  $\alpha$ -tocopherol was obtained, but from cottonseed oil a third allophanate was isolated, melting at 138–140°.

This allophanate separated as round aggregates which occasionally appeared to be made up of extremely fine thread-like needles. In this respect it differed from the  $\beta$ -allophanate, which often crystallized beautifully, even from impure solutions. A mixture of the  $\beta$  and the new allophanate gave a distinct depression of the melting point (132-136°). Like the  $\beta$ -allophanate it appeared to have a slight optical rotation, but this might have been due to

TABLE I  
*Biological Assays of Tocopherols from Different Sources*

Tocopherol	M.p. of allophanate	Source	Level fed	No. of rats	No. of lit- ters	Litter- ing	Average No. of living young per litter	Average weight	Total No. of dead young
	°C.		mg.			per cent		gm.	
$\alpha$	158-160	Wheat germ oil	3	15	13	86.6	5.6	4.9	0
	158-160	" " "	1	9	1	11.1	1.0	4.0	0
	158-160	Cottonseed oil	3	16	16	100.0	6.2	5.1	9
	158-160	" "	1	17	4	23.5	4.6	5.5	0
	158-160	Lettuce leaves	3	6	4	66.6	5.0	5.4	12
	158-160	" "	1	6	2	33.3	4.0	4.5	1
	158-160	Palm oil	3	6	5	83.3	8.2	5.4	0
	158-160	" "	1	7	2	28.6	6.0	4.2	0
$\beta$	136-138	Wheat germ oil	6	4	4	100.0	7.2	4.9	0
	136-138	" " "	3	15	2	13.3	7.5	5.4	0
	136-138	" " "	1	5	0	0.0	0.0	0.0	0
	144-146	" " "	8	5	4	80.0	4.8	5.3	10
$\gamma$	134-135*	Cottonseed oil	8	4	4	100.0	6.8	5.8	0
	134-135*	" "	3	8	4	50.0	7.2	5.0	0
	134-135*	" "	1	8	3	37.5	5.3	4.4	0
	138-140	" "	8	5	4	80.0	5.7	5.3	0
	138-140	" "	3	5	1	20.0	1.0	4.0	2

\* Impure, contained some  $\alpha$ -tocopherol.

an impurity. For this third member of the series the name  $\gamma$ -tocopherol is proposed. The free tocopherol was not potent at a level of 3 mg., but did show activity when administered in a single dose of 8 mg.

From palm oil an allophanate fraction melting at 135° was obtained, which gave no melting point depression when mixed with the slightly impure  $\gamma$ -tocopheryl allophanate of the same

melting point from cottonseed oil. Unfortunately the amount of this fraction obtained was insufficient for its complete purification, but it seemed that the tocopherol make-up of palm oil was qualitatively similar to that of cottonseed oil.<sup>1</sup>

#### EXPERIMENTAL

##### *Wheat Germ Oil*

3.5 kilos of wheat germ oil were saponified and the non-saponifiable fraction was handled as previously described (1) except that the last traces of sterols were not precipitated with digitonin, a step which, in the case of wheat germ and cottonseed oils at least, appeared to be unnecessary. The allophanates were also treated as described before, except that it was found that the crude  $\alpha$ -tocopheryl allophanate was more conveniently freed from oily impurities by recrystallization from small amounts of acetone than by recrystallization from alcohol and washing with petroleum ether.

From 3.5 kilos of wheat germ oil, 3.2 gm. of  $\alpha$ -tocopheryl allophanate, melting at 158–160°, and 1.5 gm. of  $\beta$ -allophanate, melting at 136–138°, were obtained. Since further recrystallization from alcohol effected no change in the melting point of the latter allophanate, acetone was tried. Fractions melting over a broad range, *e.g.* 138–144°, were soon obtained, which on further recrystallization melted sharply at 144–146° (uncorrected). The allophanate was much more soluble in acetone than in alcohol, so that much material remained in the mother liquors, making it necessary to work up the intermediate fractions, and rendering this further purification very laborious and time-consuming.

*Optical Rotation of  $\beta$ -Tocopheryl Allophanate*—52.6 mg. in 3 cc. of benzene, 1 dm. tube, gave a rotation of +0.10°.

$$[\alpha]_D^{25} = \frac{+0.10 \times 3 \times 1000}{52.6} = +5.7^\circ$$

*Analysis*—Found, C 71.71, 71.97; H 10.00, 10.06; N 5.53, 5.76

C<sub>31</sub>H<sub>52</sub>N<sub>2</sub>O<sub>4</sub>. Calculated. C 72.04, H 10.15, N 5.42

C<sub>30</sub>H<sub>50</sub>N<sub>2</sub>O<sub>4</sub>. “ “ 71.65, “ 10.03, “ 5.57

<sup>1</sup> We have since learned that palm oil concentrates possessing both biological activity and the absorption band at 2980 Å. have been prepared at the Iowa laboratory.

*Cottonseed Oil*

3.5 kilos of cottonseed oil were treated as described above for wheat germ oil. By recrystallization from alcohol, 300 mg. of an allophanate melting at 158–160° were obtained without difficulty. This preparation gave no melting point depression when mixed with  $\alpha$ -tocopheryl allophanate from wheat germ oil. However, on analysis, carbon values were observed about 0.5 per cent higher than those for the wheat germ oil product, but by recrystallization from acetone a preparation was obtained whose analytical values were in better agreement with those previously observed.

*Analysis of  $\alpha$ -Tocopheryl Allophanate from Cottonseed Oil*—Found, C 72.23, 72.32; H 10.09, 10.18; N 5.54

$C_{31}H_{52}N_2O_4$ . Calculated. C 72.04, H 10.15, N 5.42

*Conversion to  $p$ -Nitrophenylurethane*—This was carried out as described before for  $\alpha$ -tocopherol from wheat germ oil (1). With the original sample of  $p$ -nitrophenyl isocyanate, a  $p$ -nitrophenylurethane was obtained, melting at 129–131°, which gave no depression on mixed melting with a sample of  $\alpha$ -tocopheryl  $p$ -nitrophenylurethane from wheat germ oil. However, with a different preparation of the reagent, it was possible to secure with ease a  $p$ -nitrophenylurethane melting sharply at 133°.

*Analysis of  $p$ -Nitrophenylurethane*—Found, C 72.77, 72.67; H 9.12, 9.27; N 4.54, 4.61

$C_{26}H_{54}N_2O_6$ . Calculated. C 72.67, H 9.15, N 4.71

$C_{27}H_{56}N_2O_6$ . “ “ 72.98, “ 9.27, “ 4.60

*$\gamma$ -Tocopheryl Allophanate*—From the mother liquors of the  $\alpha$ -allophanate a much more soluble allophanate fraction, melting at 135°, was obtained. This was a mixture of  $\alpha$ -tocopheryl allophanate and the  $\gamma$ -allophanate. With small amounts of material, a separation of the two by fractional crystallization did not prove easy. At least a partial separation may be obtained by absorption on a column of calcium carbonate from benzene solution.

100 gm. of calcium carbonate, which had been dried by heating at 150° for 3 hours, were packed into a 45 mm. glass tube, and 107 mg. of the allophanate mixture in 100 cc. of benzene were poured into the top of the column, and the chromatogram developed with

benzene. The first 850 cc. of filtrate contained no allophanates; the next 900 cc. washed through 30 mg. of an allophanate which, after one recrystallization from a small amount of alcohol, melted at 150–153° and was, no doubt, slightly impure  $\alpha$ -allophanate. The next 750 cc. of solvent contained 28 mg. of material which melted at 135–137°. The material remaining in the column was eluted with a mixture of alcohol-benzene, and it also melted at 135–137°. By several recrystallizations it was possible to bring the melting point up to 136–138°.

Using very much larger amounts of material, Dr. E. Fernholz of Merck and Company secured by fractional crystallization from alcohol a  $\gamma$ -tocopheryl allophanate melting at 138–140°.

*Rotation*—53.5 mg. dissolved in 3 cc. of benzene gave an observed rotation of +0.06°, 1 dm. tube.

$$[\alpha]_D^{20} = \frac{3 \times 0.06 \times 1000}{53.5} = +3.4^\circ$$

*Analysis*—Found, C 71.91, 71.60, 71.60; H 10.09, 9.90, 10.11; N 5.58, 5.49  
 $C_{31}H_{52}N_2O_4$ . Calculated. C 72.04, H 10.15, N 5.42  
 $C_{30}H_{50}N_2O_4$ . " " 71.65, " 10.03, " 5.57

#### *Lettuce Leaves*

In preparing our concentrate from lettuce leaves, we departed slightly from the procedure of Olcott and Mattill. High boiling petroleum ether was used in the extraction in place of ethyl alcohol, and saponification was effected by refluxing for 1 hour with methanol-KOH instead of overnight with cold ethyl alcoholic KOH. This concentrate was distilled in a molecular still in very high vacuum, instead of at 0.1 mm., which enabled the vitamin to be distilled at 110–140° instead of at 190–220°. Finally the concentrate was treated with digitonin in order to remove the last traces of sterols. Although our concentrate showed considerable general absorption, there was a definite band in the region shown by the tocopherols, namely 2900 to 3000 Å.

250 pounds of fresh lettuce leaves were dried in a steam chamber, crushed, and extracted in the cold by standing overnight in high boiling petroleum ether. The solvent was filtered off and the leaves were extracted with a second portion of petroleum ether. The united filtrates were evaporated under reduced pressure,



leaving 500 gm. of a semisolid residue. This was saponified in an atmosphere of hydrogen by refluxing with 750 cc. of methanol, containing 72 gm. of KOH, and 1 gm. of hydroquinone as anti-oxidant. The cooled reaction mixture was diluted with 4 volumes of water and extracted with peroxide-free ether.

The fractionation of the non-saponifiable matter was carried out as described by Olcott and Mattill, and the concentrate so obtained was distilled in a molecular still at a pressure indicated by the MacLeod gage of  $10^{-5}$  mm. The fraction boiling between  $110-140^{\circ}$  was taken up in methanol and chilled to  $-18^{\circ}$ , which threw out additional waxy material. After the digitonin precipitation, 5 gm. of concentrate were obtained which were active as vitamin E in a single dose of 20 mg.

The conversion to the allophanate was carried out in the usual way. The isolation of  $\alpha$ -tocopherol allophanate, melting at  $158-160^{\circ}$ , was rather easily accomplished, although the yield was small.

*Analysis*--C 72.10, 72.07;<sup>2</sup> H 10.26, 9.79;<sup>2</sup> N 5.73, 5.70

Calculated for  $C_{31}H_{52}N_2O_4$ ; C 72.04, H 10.15, N 5.42

The allophanate was converted to the *p*-nitrophenylurethane as previously described. The *p*-nitrophenylurethane melted at  $132-133^{\circ}$  and gave no depression when mixed with  $\alpha$ -tocopheryl *p*-nitrophenylurethane from cottonseed oil melting at  $133^{\circ}$ .

### *Palm Oil*

3 kilos of palm oil were treated as described for the cottonseed oil and wheat germ oil, except that the final product was distilled in a molecular still, and treated with digitonin as described for the lettuce concentrate. There were thus obtained 2.1 gm. of a concentrate which showed clearly an absorption maximum at  $2980 \text{ \AA.}$ , as well as definite vitamin E activity, when administered in a single dose of 20 mg. From this concentrate, an allophanate was secured melting at  $158-160^{\circ}$ , which gave no depression when mixed with  $\alpha$ -tocopheryl allophanate from wheat germ and cottonseed oils. The analysis gave high carbon values, as had been observed in samples of the corresponding cottonseed oil product. Lack of material prevented further purification.

<sup>2</sup> Analysis by Schoeller, Berlin.

*Analysis*—C 72.65, 72.53; H 9.85, 9.77; N 5.19, 5.29

Calculated for  $C_{51}H_{82}N_2O_4$ ; C 72.04, H 10.15, N 5.42

From the mother liquors of the  $\alpha$ -allophanate there was obtained by crystallization from alcohol a fraction melting at 134–135° which gave no depression when mixed with the crude  $\gamma$ -tocopheryl allophanate of the same melting point from cottonseed oil. Lack of material prevented further purification.

#### SUMMARY

$\alpha$ -Tocopherol has been isolated by means of the allophanate from cottonseed oil, lettuce leaves, and palm oil; and from each of these sources has, within the limits of accuracy of the test, the same vitamin E activity as that originally isolated from wheat germ oil.

Two additional tocopherols have been isolated.  $\beta$ -Tocopherol from wheat germ oil was obtained as the allophanate crystallizing in well formed needles melting at 144–146°, and  $\gamma$ -tocopherol from cottonseed oil was obtained as the allophanate melting at 138–140°. These tocopherols appeared to be one-half to one-third as potent as  $\alpha$ -tocopherol. The analysis of the allophanates of these tocopherols indicate that they may contain one  $CH_2$  group less than  $\alpha$ -tocopherol, though the possibility that all three are isomeric is by no means excluded.

A tocopherol apparently identical with  $\gamma$ -tocopherol was obtained from palm oil, but the amount was insufficient for its complete purification.

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# THERMODYNAMIC PROPERTIES OF SOLUTIONS OF AMINO ACIDS AND RELATED SUBSTANCES

## III. THE IONIZATION OF ALIPHATIC AMINO ACIDS IN AQUEOUS SOLUTION FROM ONE TO FIFTY DEGREES\*

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Thermodynamic ionization constants have previously been determined for two amino acids, glycine from 10–45° by Owen (9) and alanine from 20–45° by Nims and Smith (7). The present study includes the determination of the constants for seven other aliphatic  $\alpha$ -amino acids. Certain simplifications were introduced into the procedure, which probably increased the experimental errors. The constants for alanine were redetermined to test the reliability of the modified procedure and to extend the temperature range over which the constants were known.

### *Methods and Materials*

The amino acids were all commercial synthetic products. The purification, drying, and analysis of some of the amino acids have been described in an earlier communication (10). A similar procedure was employed with the others.

*dl-Norleucine*—Purchased from Amino Acid Manufactures.<sup>1</sup> Recrystallized once from water and twice from alcohol-water mixtures. Solubility, 0.090 and 0.089 mole per 1000 gm. of water.

*dl-Leucine*—Purchased from Amino Acid Manufactures. Recrystallized once from water and twice from alcohol-water mixtures. Solubility, 0.076 and 0.076 mole per 1000 gm. of water.

*dl-Isoleucine*—Purchased from the University of Illinois. Re-

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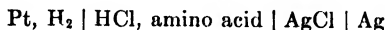
crystallized twice from water and twice from alcohol-water mixtures. Solubility, 0.165 and 0.164 mole per 1000 gm. of water.

The hydrochloric acid was prepared from a weighed amount of the constant boiling mixture, and its molality was checked by titration against recrystallized borax. The sodium hydroxide solution was prepared by adding centrifuged 50 per cent sodium hydroxide to freshly boiled distilled water and was protected from carbon dioxide. It was standardized against the hydrochloric acid. Purified sodium chloride was dried by heating in a platinum crucible and a stock solution of this prepared.

Hydrogen electrodes were of platinized platinum wire and silver-silver chloride electrodes were of the Type 2 described by Harned (2). Fresh electrodes were prepared for each solution measured. They were washed in distilled water and twice in portions of the solution to be measured. The cells were similar to those described by Nims and Smith (8). They were small enough to permit washing the electrodes and filling two cells for duplicate measurements with 30 cc. of buffer solution, an important consideration since only small amounts of some of the compounds were available. Except for the use of freshly boiled distilled water in preparing the buffer solutions, no special precautions were taken to prevent contamination with air.

### *Results and Discussion*

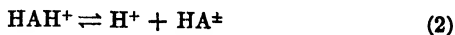
*First Acid Constant*—Buffer solutions of amino acid were measured in the cell



whose electromotive force is related to the first acid ionization constant of the amino acid by the equation

$$\begin{aligned} \text{pK}_1 - \log \frac{\gamma_{\text{HAH}^+}}{\gamma_{\text{H}^+} \gamma_{\text{HA}^\pm}} &= \frac{(E - E_0)nF}{2.3026 RT} + \log m_{\text{Cl}^-} + \log \gamma_{\text{H}^+} \gamma_{\text{Cl}^-} \\ &+ \log \frac{m_{\text{Cl}^-} - m_{\text{H}^+}}{m_{\text{A}} - m_{\text{Cl}^-} + m_{\text{H}^+}} \end{aligned} \quad (1)$$

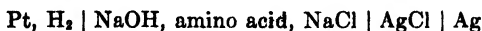
where A represents the amino acid in all forms, the ionization of which, in acid solutions, is symbolized by the equation



The other terms in Equation 1 have their usual significances, the values for  $E_0$  and  $\log \gamma_{H^+} \gamma_{Cl^-}$  being those of Harned and Ehlers (3). It is assumed that the activity of hydrochloric acid is the same as in pure hydrochloric acid solutions. Thus all the terms in Equation 1 are known except the molality of the hydrogen ion which is estimated from the equation

$$-\log m_{H^+} = \frac{(E - E_0)nF}{2.3026 RT} + \log m_{Cl^-} + \log \gamma_{H^+} \gamma_{Cl^-} \quad (3)$$

*Second Acid Constant*—Buffer solutions of amino acid were measured in the cell



whose electromotive force is related to the second acid ionization constant of the amino acid by the equation

$$\begin{aligned} pK_2 + \log \frac{\gamma_{A^-}}{\gamma_{HA^+} \gamma_{Cl^-}} &= \frac{(E - E_0)nF}{2.3026 RT} + \log m_{Cl^-} \\ &+ \log \frac{m_A - m_{NaOH} + m_{OH^-}}{m_{NaOH} - m_{OH^-}} \end{aligned} \quad (4)$$

the ionization of the amino acid in alkaline solutions being symbolized by the equation



All the terms in Equation 4 are known except  $m_{OH^-}$  which is estimated from the equation

$$\log m_{OH^-} = \log K_w + \frac{(E - E_0)nF}{2.3026 RT} + \log m_{Cl^-} \quad (6)$$

$\log K_w$  is given by the measurements of Harned and Hamer (5).

Tables I and II are a compilation of the compositions of the buffer solutions and of the observed potentials. Values for the first acid ionization constants are obtained by a plot of the right-hand side of Equation 1 against the ionic strength, for the second acid ionization constants by a plot of the right-hand side of Equation 4 against the ionic strength. The intercepts at 0 ionic strength are  $pK_1$  and  $pK_2$  respectively. A summary of these values

TABLE I

*Experimental Data Used in Determining First Acid Ionization Constant of Amino Acids*

Molality		Observed $E$ , corrected to 1 atmosphere $H_2$ at				
Amino acid	HCl	1.0°	12.5°	25.0°	37.5°	50.0°
<i>dl</i> -Alanine						
0.01985	0.01073	0.4884	0.4915	0.4943	0.4964	0.4984
0.03151	0.01806	0.4717	0.4739	0.4759	0.4776	0.4788
0.04008	0.02003	0.4731	0.4753	0.4773	0.4789	0.4802
0.06031	0.02986	0.4629	0.4654	0.4659	0.4670	0.4679
0.07764	0.03991	0.4542	0.4554	0.4565	0.4573	0.4578
0.1519	0.07533	0.4411	0.4417	0.4421	0.4422	0.4421
0.1958	0.1004	0.4335	0.4337	0.4337	0.4336	0.4332
<i>dl</i> - $\alpha$ -Amino- <i>n</i> -butyric acid						
0.02107	0.01009	0.4902	0.4937	0.4971	0.5000	0.5024
0.04111	0.01947	0.4717	0.4744	0.4769	0.4790	0.4806
0.08042	0.03964	0.4526	0.4544	0.4559	0.4572	0.4580
0.1030	0.05000	0.4470	0.4485	0.4498	0.4508	0.4515
0.1214	0.06000	0.4429	0.4441	0.4452	0.4460	0.4464
0.1415	0.07015	0.4386	0.4398	0.4408	0.4415	0.4419
0.2017	0.1005	0.4310	0.4318	0.4322	0.4325	0.4326
<i>dl</i> - $\alpha$ -Amino- <i>n</i> -valeric acid						
0.02557	0.01288	0.4831	0.4860	0.4888	0.4912	0.4932
0.02857	0.01542	0.4770	0.4789	0.4814	0.4833	0.4848
0.03946	0.01953	0.4720	0.4745	0.4768	0.4788	0.4803
0.06059	0.02941	0.4620	0.4640	0.4658	0.4672	0.4684
0.08612	0.04169	0.4535	0.4556	0.4565	0.4574	0.4582
0.1702	0.08557	0.4356	0.4363	0.4368	0.4370	0.4370
<i>dl</i> -Norleucine						
0.03205	0.01754	0.4724	0.4750	0.4772	0.4792	0.4804
0.03776	0.02005	0.4699	0.4721	0.4744	0.4761	0.4775
0.05334	0.02494	0.4681	0.4702	0.4723	0.4740	0.4753
0.06779	0.03315	0.4592	0.4611	0.4628	0.4641	0.4650
0.1036	0.04947	0.4513	0.4522	0.4534	0.4544	0.4549
0.1374	0.06281	0.4467	0.4478	0.4491	0.4500	0.4504

TABLE I—*Concluded*

Molality		Observed $F$ , corrected to 1 atmosphere $H_2$ at				
Amino acid	HCl	1.0°	12.5°	25.0°	37.5°	50.0°
<i><math>\alpha</math>-Aminoisobutyric acid</i>						
0.02073	0.00908	0.4988	0.5022	0.5056	0.5085	0.5111
0.02603	0.01405	0.4806	0.4835	0.4860	0.4882	0.4901
0.02621	0.01357	0.4825	0.4854	0.4882	0.4906	0.4925
0.03186	0.01786	0.4722	0.4747	0.4770	0.4789	0.4803
0.04184	0.01916	0.4772	0.4797	0.4817	0.4837	0.4853
0.07967	0.03960	0.4563	0.4578	0.4590	0.4603	0.4613
0.1231	0.06004	0.4474	0.4484	0.4491	0.4498	0.4502
0.1627	0.08007	0.4402	0.4410	0.4417	0.4421	0.4422
<i>dl-Valine</i>						
0.03229	0.01764	0.4700	0.4730	0.4757	0.4780	0.4797
0.03878	0.01911	0.4706	0.4734	0.4761	0.4782	0.4800
0.06114	0.02931	0.4598	0.4624	0.4646	0.4664	0.4678
0.08123	0.03783	0.4546	0.4568	0.4588	0.4604	0.4617
0.1012	0.05029	0.4458	0.4475	0.4489	0.4502	0.4510
0.1641	0.07982	0.4353	0.4368	0.4379	0.4387	0.4392
0.1985	0.1004	0.4291	0.4300	0.4308	0.4314	0.4317
<i>dl-Leucine</i>						
0.02809	0.01579	0.4745	0.4772	0.4797	0.4818	0.4832
0.03298	0.01934	0.4678	0.4701	0.4722	0.4740	0.4753
0.04041	0.02140	0.4678	0.4701	0.4722	0.4739	0.4752
0.04953	0.02509	0.4654	0.4675	0.4693	0.4708	0.4720
0.06315	0.03129	0.4608	0.4624	0.4639	0.4652	0.4662
0.09959	0.04969	0.4488	0.4501	0.4512	0.4521	0.4526
<i>dl-Isoleucine</i>						
0.02961	0.01916	0.4640	0.4666	0.4690	0.4708	0.4723
0.03854	0.02022	0.4687	0.4712	0.4736	0.4757	0.4774
0.05216	0.02479	0.4665	0.4690	0.4714	0.4733	0.4750
0.06720	0.03356	0.4573	0.4594	0.4614	0.4631	0.4644
0.09932	0.05007	0.4475	0.4492	0.4507	0.4519	0.4528
0.1468	0.06985	0.4419	0.4433	0.4446	0.4456	0.4463



TABLE II  
*Experimental Data Used in Determining Second Acid Ionization Constant of Amino Acids*

Molality			Observed <i>E</i> , corrected to 1 atmosphere H <sub>2</sub> at				
Amino acid	NaOH	NaCl	1.0°	12.5°	25.0°	37.5°	50.0°
<i>dl</i> -Alanine							
0.01960	0.01054	0.00972	0.9252	0.9275	0.9291	0.9307	0.9314
0.02128	0.00995	0.00956	0.9193	0.9214	0.9230	0.9241	0.9248
0.02640	0.01382	0.01387	0.9154	0.9177	0.9194	0.9206	0.9211
0.03196	0.01529	0.01555	0.9097	0.9113	0.9121	0.9128	0.9127
0.03998	0.01991	0.02026	0.9051	0.9069	0.9081	0.9088	0.9088
0.06258	0.03047	0.02968	0.8956	0.8970	0.8978	0.8979	0.8975
0.08276	0.04099	0.03943	0.8900	0.8910	0.8916	0.8914	0.8906
<i>dl</i> - $\alpha$ -Amino- <i>n</i> -butyric acid							
0.01645	0.00789	0.00765	0.9220	0.9245	0.9267	0.9283	0.9294
0.02547	0.01207	0.01486	0.9054	0.9074	0.9090	0.9098	0.9101
0.03678	0.01800	0.03192	0.8886	0.8898	0.8906	0.8908	0.8903
0.04245	0.02175	0.02106	0.9010	0.9028	0.9039	0.9045	0.9044
0.05206	0.02613	0.02569	0.8957	0.8971	0.8978	0.8981	0.8975
0.08593	0.04455	0.04393	0.8848	0.8858	0.8862	0.8860	0.8850
<i>dl</i> - $\alpha$ -Amino- <i>n</i> -valeric acid							
0.01596	0.00876	0.00799	0.9269	0.9294	0.9317	0.9333	0.9343
0.01767	0.00917	0.00880	0.9215	0.9238	0.9260	0.9273	0.9279
0.01904	0.01042	0.00994	0.9216	0.9239	0.9259	0.9272	0.9279
0.01916	0.01063	0.01818	0.9086	0.9104	0.9120	0.9127	0.9131
0.02548	0.01358	0.01347	0.9131	0.9151	0.9168	0.9178	0.9182
0.05205	0.02537	0.02515	0.8952	0.8966	0.8974	0.8975	0.8969
0.08060	0.04085	0.04004	0.8869	0.8878	0.8879	0.8876	0.8866
<i>dl</i> -Norleucine							
0.02272	0.01106	0.01115	0.9152	0.9171	0.9186	0.9194	0.9197
0.03084	0.01509	0.01574	0.9074	0.9091	0.9102	0.9107	0.9105
0.04205	0.01976	0.02020	0.9000	0.9015	0.9023	0.9025	0.9021
0.04883	0.02477	0.02545	0.8987	0.8997	0.9004	0.9008	0.9003
0.09032	0.04600	0.04753	0.8846	0.8853	0.8854	0.8847	0.8832

TABLE II—*Concluded*

Molality			Observed $E$ , corrected to 1 atmosphere $H_2$ at				
Amino acid	NaOH	NaCl	1.0°	12.5°	25.0°	37.5°	50.0°
<i><math>\alpha</math>-Aminoisobutyric acid</i>							
0.02351	0.01039	0.00962	0.9360	0.9379	0.9392	0.9399	0.9402
0.03185	0.01465	0.01509	0.9272	0.9284	0.9292	0.9296	0.9293
0.04684	0.01968	0.01948	0.9178	0.9189	0.9193	0.9198	0.9186
0.04916	0.02140	0.02116	0.9170	0.9179	0.9183	0.9184	0.9177
0.05934	0.02637	0.02401	0.9152	0.9159	0.9162	0.9160	0.9150
0.09303	0.04636	0.04711	0.9051	0.9055	0.9048	0.9043	0.9032
<i>dl-Valine</i>							
0.02029	0.00854	0.00992	0.9040	0.9061	0.9075	0.9084	0.9085
0.03184	0.01442	0.01571	0.8960	0.8978	0.8991	0.8998	0.8998
0.04044	0.01954	0.02044	0.8929	0.8945	0.8956	0.8962	0.8962
0.05010	0.02381	0.02406	0.8881	0.8896	0.8906	0.8910	0.8906
0.06086	0.02964	0.03018	0.8841	0.8852	0.8858	0.8858	0.8853
0.1032	0.04924	0.04423	0.8747	0.8756	0.8758	0.8753	0.8740
<i>dl-Leucine</i>							
0.01653	0.00870	0.00931	0.9176	0.9199	0.9216	0.9228	0.9233
0.02128	0.01122	0.00918	0.9182	0.9204	0.9219	0.9235	0.9243
0.02652	0.01412	0.01351	0.9096	0.9118	0.9133	0.9143	0.9144
0.03339	0.01874	0.01746	0.9068	0.9088	0.9096	0.9106	0.9106
0.06994	0.03652	0.03434	0.8882	0.8891	0.8889	0.8882	0.8871
<i>dl-Isoleucine</i>							
0.01931	0.01061	0.00864	0.9222	0.9245	0.9266	0.9280	0.9291
0.02313	0.01197	0.00915	0.9176	0.9198	0.9220	0.9230	0.9234
0.02440	0.01349	0.01330	0.9125	0.9145	0.9161	0.9171	0.9177
0.03073	0.01565	0.01606	0.9039	0.9059	0.9073	0.9079	0.9060
0.03390	0.01900	0.01762	0.9064	0.9082	0.9095	0.9103	0.9106
0.05263	0.02527	0.02594	0.8907	0.8919	0.8927	0.8918	0.8905
0.09108	0.04697	0.04682	0.8805	0.8811	0.8810	0.8795	0.8774

appears in Tables III and IV. Figs. 1 and 2 illustrate the curves obtained from the results at 25°.

The average agreement of the potentials in the two cells used for each point was approximately  $\pm 0.0001$  volt, both in the acid

TABLE III  
*Thermodynamic Functions for Ionization of Aliphatic Amino Acids*

	1.0°	12.5°	25.0°	37.5°	50.0°
<i>dl</i> -Alanine					
pK <sub>1</sub>	2.426	2.383	2.348	2.330	2.332
" *	2.426	2.382	2.350	2.333	2.331
Δ <i>F</i> <sub>1</sub>	3040	3110	3200	3320	3450
Δ <i>H</i> <sub>1</sub>	1510	1210	800	320	-250
Δ <i>S</i> <sub>1</sub>	-5.6	-6.6	-8.0	-9.6	-11.4
<i>dl</i> -α-Amino- <i>n</i> -butyric acid					
pK <sub>1</sub>	2.334	2.310	2.286	2.288	2.297
" *	2.335	2.305	2.288	2.286	2.300
Δ <i>F</i> <sub>1</sub>	2930	3010	3120	3250	3400
Δ <i>H</i> <sub>1</sub>	1090	750	310	-220	-830
Δ <i>S</i> <sub>1</sub>	-6.7	-7.9	-9.4	-11.2	-13.1
<i>dl</i> -α-Amino- <i>n</i> -valeric acid					
pK <sub>1</sub>	2.376	2.340	2.318	2.309	2.313
" *	2.378	2.341	2.316	2.308	2.314
Δ <i>F</i> <sub>1</sub>	2980	3060	3160	3280	3420
Δ <i>H</i> <sub>1</sub>	1290	970	550	50	-540
Δ <i>S</i> <sub>1</sub>	-6.1	-7.3	-8.8	-10.4	-12.3
<i>dl</i> -Norleucine					
pK <sub>1</sub>	2.394	2.356	2.335	2.324	2.328
" *	2.398	2.355	2.333	2.324	2.329
Δ <i>F</i> <sub>1</sub>	3010	3080	3180	3300	3440
Δ <i>H</i> <sub>1</sub>	1300	980	560	60	-540
Δ <i>S</i> <sub>1</sub>	-6.3	-7.3	-8.8	-10.4	-12.3
α-Aminoisobutyric acid					
pK <sub>1</sub>	2.419	2.380	2.357	2.351	2.356
" *	2.424	2.381	2.359	2.350	2.355
Δ <i>F</i> <sub>1</sub>	3040	3110	3220	3340	3480
Δ <i>H</i> <sub>1</sub>	1300	980	560	60	-540
Δ <i>S</i> <sub>1</sub>	-6.2	-7.1	-8.9	-10.6	-12.4

TABLE III—*Concluded*

	1.0°	12.5°	25.0°	37.5°	50.0°
<i>dl</i> -Valine					
pK <sub>1</sub>	2.320	2.297	2.286	2.292	2.310
" *	2.323	2.298	2.287	2.292	2.313
Δ <i>F</i> <sub>1</sub>	2910	3000	3120	3260	3420
Δ <i>H</i> <sub>1</sub>	890	540	80	-460	-1100
Δ <i>S</i> <sub>1</sub>	-7.3	-8.6	-10.2	-12.0	-13.9
<i>dl</i> -Leucine					
pK <sub>1</sub>	2.383	2.348	2.328	2.327	2.333
" *	2.387	2.350	2.329	2.326	2.335
Δ <i>F</i> <sub>1</sub>	2990	3070	3180	3300	3450
Δ <i>H</i> <sub>1</sub>	1180	860	420	-90	-700
Δ <i>S</i> <sub>1</sub>	-6.5	-7.7	-9.2	-10.9	-12.8
<i>dl</i> -Isoleucine					
pK <sub>1</sub>	2.365	2.338	2.318	2.317	2.332
" *	2.369	2.337	2.320	2.318	2.332
Δ <i>F</i> <sub>1</sub>	2970	3050	3160	3290	3450
Δ <i>H</i> <sub>1</sub>	1080	740	300	-220	-840
Δ <i>S</i> <sub>1</sub>	-6.9	-8.1	-9.6	-11.3	-13.3

\* From Equation 7.

and the alkaline solutions. The results in the acid solutions are probably consistent to within  $\pm 0.005$  pK, the results in the alkaline solutions being perhaps less accurate because of the curvature with ionic strength (see Fig. 2).

Harned and Embree (4) found that the variation with temperature of the ionization constants of many weak acids could be represented by an equation which, in a modified form, is

$$\text{pK} = \text{pK}_{\text{max.}} + p(t - \theta)^2 \quad (7)$$

where  $\theta$  is the temperature of maximum ionization and  $\text{pK}_{\text{max.}}$  the constant at that temperature. The constants  $\text{pK}_{\text{max.}}$  and  $\theta$  thus become valuable constants characterizing an acid. As these authors suggest, for any correlation of the ionization constants with constitution to have real value it is essential to select the

TABLE IV  
*Thermodynamic Functions for Ionization of Aliphatic Amino Acids*

	1.0°	12.5°	25.0°	37.5°	50.0°
<i>dl</i> -Alanine					
$pK_2$	10.586	10.225	9.866	9.548	9.256
" *	10.581	10.227	9.872	9.548	9.256
$\Delta F_2$	13270	13360	13460	13560	13680
$\Delta H_2$	10990	11080	11040	10890	10580
$\Delta S_2$	-8.3	-8.0	-8.0	-8.6	-9.6
<i>dl</i> - $\alpha$ -Amino- <i>n</i> -butyric acid					
$pK_2$	10.530	10.180	9.830	9.518	9.234
" *	10.527	10.181	9.834	9.519	9.234
$\Delta F_2$	13200	13300	13410	13520	13650
$\Delta H_2$	10750	10820	10770	10580	10260
$\Delta S_2$	-9.0	-8.7	-8.8	-9.4	-10.5
<i>dl</i> - $\alpha$ -Amino- <i>n</i> -valeric acid					
$pK_2$	10.508	10.154	9.808	9.490	9.198
" *	10.507	10.153	9.808	9.489	9.202
$\Delta F_2$	13180	13270	13380	13480	13600
$\Delta H_2$	10840	10910	10880	10700	10380
$\Delta S_2$	-8.5	-8.2	-8.4	-9.0	-9.9
<i>dl</i> -Norleucine					
$pK_2$	10.546	10.190	9.834	9.513	9.224
" *	10.544	10.190	9.837	9.514	9.223
$\Delta F_2$	13220	13310	13420	13520	13630
$\Delta H_2$	10950	11030	11050	10840	10540
$\Delta S_2$	-8.3	-8.0	-7.9	-8.6	-9.6
$\alpha$ -Aminoisobutyric acid					
$pK_2$	10.960	10.580	10.205	9.872	9.561
" *	10.956	10.585	10.212	9.870	9.560
$\Delta F_2$	13740	13830	13930	14020	14130
$\Delta H_2$	11480	11600	11610	11510	11260
$\Delta S_2$	-8.2	-7.8	-7.7	-8.1	-8.9

TABLE IV—*Concluded*

	1.0°	12.5°	25.0°	37.5°	50.0°
<i>dl</i> -Valine					
pK <sub>2</sub>	10.413	10.064	9.719	9.405	9.124
" *	10.413	10.067	9.721	9.406	9.123
Δ <i>F</i> <sub>2</sub>	13060	13150	13260	13360	13490
Δ <i>H</i> <sub>2</sub>	10370	10800	10740	10560	10230
Δ <i>S</i> <sub>2</sub>	-8.5	-8.2	-8.4	-9.0	-10.1
<i>dl</i> -Leucine					
pK <sub>2</sub>	.095	9.744	9.434	9.142	
" *	.100	9.750	9.434	9.142	
Δ <i>F</i> <sub>2</sub>		13300	13400	13510	
Δ <i>H</i> <sub>2</sub>		10900	10730	10410	
Δ <i>S</i> <sub>2</sub>	.9	-8.0	-8.6	-9.6	
<i>dl</i> -Isoleucine					
	.100	9.758	9.439	9.157	
	.109	9.761	9.443	9.158	
		13310	13420	13530	
		10830	10650	10330	
	.1	-8.3	-8.9	-9.9	

\* From Equation 7.

values of the ionization constants at their maxima. The value for  $p$  used by Harned and Embree (4) was  $5 \times 10^{-5}$ ; this value was found to be satisfactory for the first acid constants, but a larger figure,  $10 \times 10^{-5}$ , gave better agreement with the second acid constants. A plot of  $(\log K + pt^2)$  against  $t$  should give a straight line, the slope of which is  $2 p \theta$ , and the intercept at  $0^\circ$  is  $(\log K_m - p \theta^2)$ . Figs. 3 and 4 represent plots of this for the amino acid constants, and Table V is a compilation of the values for  $pK_{\max.}$  and for  $\theta$ . The deviations of the determined constants from a straight line are within the experimental error. The values for the second constant are less reliable than those for the first since  $\theta_2$  is far beyond the highest temperature at which the constants were determined.

The changes in free energy, heat content, and entropy may be

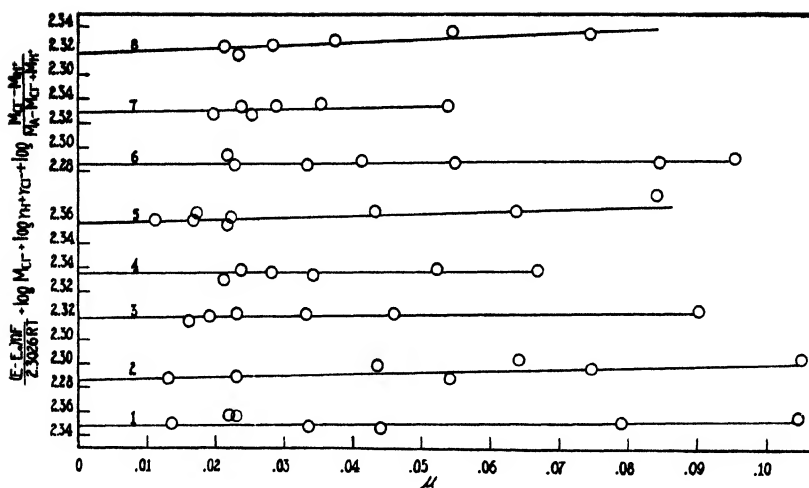


FIG. 1. A plot of the right-hand side of Equation 1 against the ionic strength. The intercept at 0 ionic strength is  $pK_1$ . Curve 1 represents *dl*-alanine; Curve 2, *dl*- $\alpha$ -amino-*n*-butyric acid; Curve 3, *dl*- $\alpha$ -amino-*n*-valeric acid; Curve 4, *dl*-norleucine; Curve 5,  $\alpha$ -aminoisobutyric acid; Curve 6, *dl*-valine; Curve 7, *dl*-leucine; Curve 8, *dl*-isoleucine.

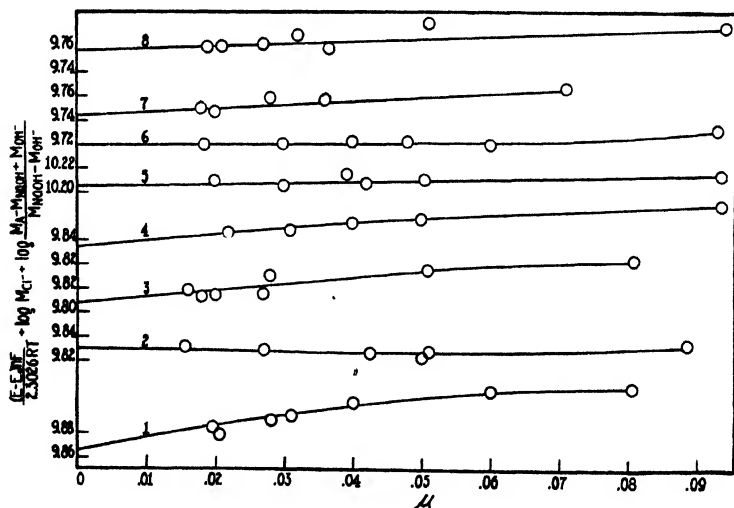
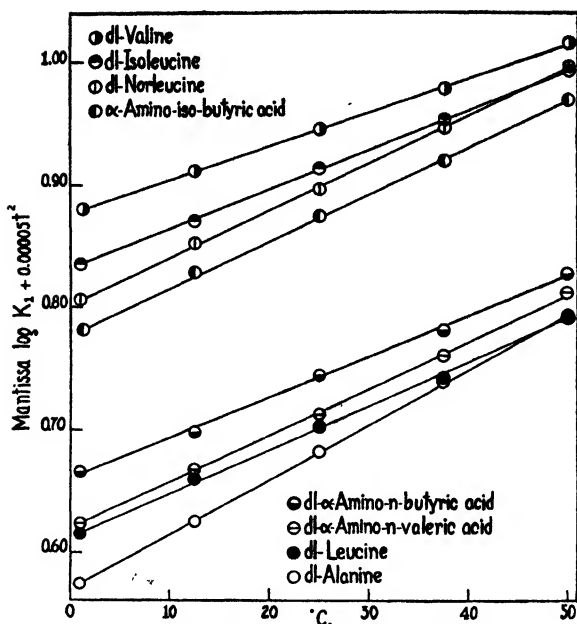


FIG. 2. A plot of the right side of Equation 4 against the ionic strength. The intercept at 0 ionic strength is  $pK_2$ . Curve 1 represents *dl*-alanine; Curve 2, *dl*- $\alpha$ -amino-*n*-butyric acid; Curve 3, *dl*- $\alpha$ -amino-*n*-valeric acid; Curve 4, *dl*-norleucine; Curve 5,  $\alpha$ -aminoisobutyric acid; Curve 6, *dl*-valine; Curve 7, *dl*-leucine; Curve 8, *dl*-isoleucine.

FIG. 3. A plot of  $\log K_1 + pl^2$  against  $t$ 

calculated from the following equations, obtained from Equation 7 and the proper thermodynamic definitions.

$$\Delta F_1 = 2.3026 RT \text{ p}K_1 \quad (8)$$

$$\Delta H_1 = -2.3026 \times 10^{-4} RT^2 (t - \theta_1) \quad (9)$$

$$\Delta H_2 = -4.6052 \times 10^{-4} RT^2 (t - \theta_2) \quad (10)$$

$$\Delta S_1 = \frac{\Delta H_1 - \Delta F_1}{T} \quad (11)$$

The equations for  $\Delta F_2$  and for  $\Delta S_2$  are similar to Equations 8 and 11. These quantities are given in Tables III and IV.

The results agree, within moderate limits, with those obtained in cells with liquid junctions (1, 6), although the first acid constant is usually slightly lower and the second acid constant approximately 0.1 pK higher than the apparent constants. The constants previously determined for alanine (7) at 25° were pK<sub>1</sub> 2.340, pK<sub>2</sub> 9.870, compared with the values in Tables III and IV of 2.348 and 9.866.



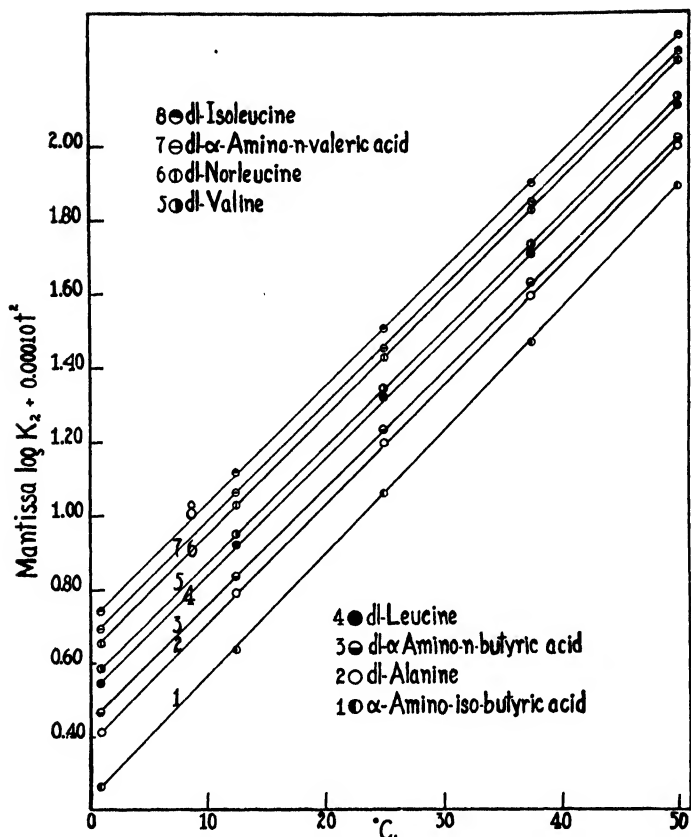


FIG. 4. A plot of  $\log K_2 + pt^2$  against  $t$ . For convenience in plotting, 0.2 has been added to the functions for  $\alpha$ -aminoisobutyric acid,  $dl$ -norleucine,  $dl$ - $\alpha$ -amino- $n$ -valeric acid, and  $dl$ -isoleucine.

TABLE V

*Maxima of First and Second Acid Ionization Constants and Temperatures of Maximum Ionization of Amino Acids*

Amino acid	$pK_1$ max.	$\theta_1$	$pK_2$ max.	$\theta_2$
$dl$ -Alanine.....	2.330	44.8	8.028	160.8
$dl$ - $\alpha$ -Amino- $n$ -butyric acid.....	2.285	32.6	8.081	157.4
$dl$ - $\alpha$ -Amino- $n$ -valeric ".....	2.307	38.6	8.020	158.7
$dl$ -Norleucine.....	2.323	38.8	8.006	160.3
$\alpha$ -Aminoisobutyric acid.....	2.349	38.8	8.170	167.9
$dl$ -Valine.....	2.287	27.0	7.976	157.1
$dl$ -Leucine.....	2.324	35.4	7.954	159.0
$dl$ -Isoleucine.....	2.317	32.4	7.989	158.1

In spite of the fact that thermodynamic constants are now available for nine aliphatic amino acids, no quantitative relationships are apparent. The range of the constants is small, as is to be expected since the acids differ only in the number and arrangement of methylene groups. The compound,  $\alpha$ -aminoisobutyric acid has both the highest  $pK_{1max}$ . and  $pK_{2max}$ . Whether this is related to the presence of a tertiary carbon cannot be decided, since no other like substances have been studied.  $\alpha$ -Aminoisobutyric acid also has a much higher value for  $\theta_2$  than has any of the other amino acids. At first the values for  $\theta_1$  tend to decrease with an increase in the number of methylene groups as do the fatty acids (4). Glycine (4) and alanine have appreciably higher values for  $\theta_1$  than the others, but  $\theta_1$  for valine is much lower than for the leucines.

#### SUMMARY

The thermodynamic ionization constants of *dl*-alanine, *dl*- $\alpha$ -amino-*n*-butyric acid, *dl*- $\alpha$ -amino-*n*-valeric acid, *dl*-norleucine,  $\alpha$ -aminoisobutyric acid, *dl*-valine, *dl*-leucine, and *dl*-isoleucine have been determined from 1–50° from electromotive force measurements of cells without liquid junctions.

From these data the values for the maximum ionization, temperature of maximum ionization, and changes in free energy, heat content, and entropy due to the ionization process have been calculated.

We thank Professors H. S. Harned and D. I. Hitchcock for valuable advice and criticism.

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## THE HYDROLYSIS OF GLYCERIDES BY CRUDE PANCREAS LIPASE\*

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Of the ordinary fats, tristearin appears to be the most refractory toward enzymic hydrolysis *in vitro*. By present methods for the demonstration of lipase activity this fat is hydrolyzed very slowly when at all. The most rapid hydrolysis by enzymic means that has been seen in the literature was reported by Davis (1) with a commercial lipase preparation. This author obtained about 14 per cent splitting in 12 hours at 37°. Other investigators have not nearly approached this figure. A technique capable of splitting tristearin easily might therefore be expected to give a more complete picture of the range of lipase activity than can be obtained by other methods.

Tristearin can be rapidly and completely hydrolyzed in the presence of bile, when under optimal conditions in regard to temperature, emulsification, and elimination of the inhibitory—perhaps synthetic—effects of the end-products of digestion. Hydrolysis is very slow at temperatures below 30° and only between 40–50° is the splitting comparable in speed to that of other fats. At 50° destruction of the enzyme also takes place.

Permanent emulsification of the substrate may be secured by dissolving it in a solution of glycerol and dried bile, then diluting the glycerol with water, buffer salts, and the other required ingredients. Glycerol in moderate concentrations is known not to inhibit lipase activity seriously.

By including in the system sufficient calcium chloride to form calcium stearate with all the stearic acid liberated in the course

\* Food Research Division Contribution No. 324.

of the hydrolysis, the speed of that reaction is decidedly increased. Too much calcium chloride is somewhat inhibitory.

The enzyme used in this work was prepared from pig pancreas by the usual method of soaking the minced glands first in acetone, then in acetone and ether, then in ether, and finally drying in air. The finely powdered and sieved gland was stirred with 17 times its weight of 87 per cent glycerol, and the lipase-containing glycerol extract filtered off.

*Method for Lipase in Detail*—0.00565 mole of substrate (0.5 gm. when olive oil is used) is weighed into a glass-stoppered bottle of about 125 cc. capacity. To this are added 5 cc. of a solution of dried ox bile in glycerol<sup>1</sup> kept at 60–70° for convenience in measuring. A quantity of glass beads is introduced, and the bottle is placed in boiling water and shaken until the substrate is completely dissolved. The bottle is then shaken under the cold water tap until the contents are cooled down to room temperature. The fat should now be dissolved or emulsified in the glycerol and the mixture should be viscous, but not solid. If the fat melts at so high a temperature that the contents of the bottle are solid at room temperature, they should be warmed slightly.

To the emulsion of glycerol and fat are then added 10 cc. of 0.05 M ammonium chloride-ammonia buffer at pH 8.0, 100 mg. of calcium chloride (in water solution), and 0.25 cc. of 3 per cent phenolphthalein solution (or other indicator). Enough water is next added so that the total volume of the system will be 30.0 cc. after the enzyme has been introduced. The enzyme is put in last of all and the contents of the bottle are well mixed, but not shaken to a froth. A 5 cc. sample of the emulsion is pipetted into 75 cc. of a mixture of 9 volumes of alcohol and 1 volume of ether and titrated with alcoholic KOH, with phenolphthalein, o-cresolphthalein, or thymolphthalein as an indicator.

<sup>1</sup> Prepared by adding to the dried bile an equal weight of water and heating the solution for 1 to 2 hours in the autoclave at 15 pounds pressure. 10 cc. of glycerol are then added for each gm. of dried bile and the mixture is heated on the steam bath until a clear solution is obtained. This may require several hours. The glycerol-bile solution is kept stoppered in the ice box, and warmed up before it is used. It contains no esterase or lipase and only traces of proteins digestible by pancreas extracts.

Since Willstätter and Waldschmidt-Leitz (2) have shown that in strong alcohol ammonia is practically without influence on titrations made with basic indicators, it follows that the pH of the aqueous digestion system may be continuously adjusted by the addition of ammonia without materially changing the values obtained by alcoholic titration. In order to minimize the error introduced by increasing the volume, small amounts of strong ammonia (5 N) are added until the indicator shows the desired shade of color. The pH is kept within a fairly constant range by the occasional addition of more ammonia during the digestion.

Although apparently crude, the method permits practically complete splitting of the simple triglycerides from tricaproin to and including tristearin, as well as of monostearin and triolein. We have also used this scheme in miniature for determining the saponification numbers of fats, using for the analysis about 150 mg. of material. Controls containing enzyme and bile but no fat, and others containing only enzyme and buffer, show that neither lipolysis nor proteolysis causes a significant error in the results, although with experiments that run over 3 or 4 hours proteolytic effects are not entirely negligible. The enzyme preparations are practically fat-free. The emulsion appears to be sufficiently uniform to permit removal of aliquot portions by a pipette. Repeated trials have shown that the titration readings on several samples taken almost simultaneously during a digestion vary at most by 0.03 cc. of 0.1 N alkali; that is, about the allowable error in the titration itself. Furthermore, duplicate experiments agree very closely. When no enzyme is present in the system, the values obtained in the titration of aliquot portions remain unchanged for at least 24 hours, as does the appearance of the emulsion. Irregularities in the pH of the system are unavoidable but may be minimized by adding the ammonia at intervals as short as the operator cares to make them. Lipase does not appear to have a very sharp pH optimum. Experiments with tristearin at pH 8.2 to 8.4 (phenolphthalein) and at 7.2 to 7.5 (cresol red) showed very little difference in the velocity of splitting. The method has the advantages of rapidity, general applicability to lipoidal substrates, flexibility as to the approximate pH of the digestion, and absence of large quantities of buffer salts.

In order to give an idea of the magnitude of the errors due to undesired enzymic activities in the system, the results of a series of controls are given in Table I. The enzyme was used in the amount of 2.0 cc. of glycerol extract in a total volume of 30 cc. The corrections are small for short digestion periods, but are included in the computation of monomolecular reaction constants, shown later.

*Course of Stearin Hydrolysis*—With the proportions of reagents (except enzyme) as outlined, the course of stearin hydrolysis was observed in several experiments in which larger volumes were employed. The results are shown in Table II for tristearin and for monostearin.

TABLE I  
*Control Experiments*

Time	0.1 N KOH		
	System without enzyme (showing lipolytic effect of bile)	System without substrate (showing effect of enzyme on bile and on itself)	Enzyme plus buffer only
<i>hrs.</i>	<i>cc.</i>	<i>cc.</i>	<i>cc.</i>
0.5	0	0	
1	0		
2	0	0.02	
3	0	0.10	-0.02
7	0	0.09	+0.03
10		0.14	
13	+0.10	0.30	

With tristearin the reaction speed approximates a monomolecular equation.

The numerical values of this monomolecular constant are also roughly proportional to the amounts of enzyme used. It is quite evident, however, that the reaction moves fastest at the start. The hydrolysis of monostearin under the same conditions is more nearly monomolecular, and the numerical value of the computed constants is a fairly acceptable measure of the amount of enzyme present. Speculation on the mode of lipase action based on the rate of hydrolysis is unfortunately handicapped by the probability that the size of the fat particles is not uniform. That the smallest particles are hydrolyzed first is the simplest

TABLE II

*Hydrolysis of Tristearin and Monostearin*

$kt = 2 - \log(100 - x)$ ;  $x^*$  = per cent hydrolysis;  $t$  = time in minutes. Hydrolysis was carried out at 40°, pH 8.2 to 8.5.

Tristearin											
Experiment 1			Experiment 2			Experiment 3			Experiment 4		
$t$	$x$	$k$	$t$	$x$	$k$	$t$	$x$	$k$	$t$	$x$	$k$
25	35	0.0075	5	11	0.0100	15	19	0.0061	20	12	0.0028
50	53	0.0066	30	30	0.0052	30	29	0.0050	60	25	0.0021
65	63	0.0066	50	39	0.0043	45	38	0.0046	100	35	0.0019
75	69	0.0068	80	51	0.0039	60	45	0.0043	160	42	0.0015
85	73	0.0067	110	61	0.0037	105	59	0.0037	220	51	0.0014
95	78	0.0069	125	65	0.0037	120	64	0.0037	340	63	0.0013
120	87	0.0074	135	68	0.0037	130	65	0.0035	370	64	0.0012
126	89	0.0076	145	72	0.0038	140	68	0.0035	400	66	0.0012
			160	76	0.0039	150	69	0.0034	515	72	0.0011
			180	79	0.0038	165	73	0.0035	640	75	0.0009
			200	83	0.0039	180	78	0.0036			
						200	81	0.0036			
						220	89	0.0044			
Relative enzyme quantity†..		8			4			4			1

## Monostearin

Experiment 5			Experiment 6			Experiment 7		
$t$	$x$	$k$	$t$	$x$	$k$	$t$	$x$	$k$
15	31	0.0107	25	27.4	0.0056	30	10	0.0015
25	43	0.0098	41	41	0.0056	80	20	0.0012
40	58	0.0094	61	51	0.0051	138	31	0.0012
67	82	0.0110	93	67	0.0052	186	38	0.0011
93	99		144	84	0.0055	400	55	0.0009
Relative enzyme quantity†.....		8			4			1

\* The actual titration differences ( $\Delta$ ) in cc. of 0.1 N KOH can be calculated from  $x = 100 \Delta / 2.83$  for the triglyceride and from  $x = 100 \Delta / 0.94$  for the monoglyceride.

† Relative enzyme quantity 1 represents 0.50 cc. of pancreas extract in 30 cc. of digestion mixture.



explanation of the high initial rate. There is also some evidence that the reaction tends to go faster near the end, but it must be remembered that errors of measurement are greatly multiplied in the computations as the amount of undigested substrate becomes small.

It is possible that the decomposition of the individual tristearin molecule takes place in steps, first to distearin, then to monostearin. There is then the further possibility that a large part of the unhydrolyzed substrate might exist as distearin at the time of one-third total hydrolysis, or as monostearin at the time of two-thirds hydrolysis. Thus the lipase would be attacking a substrate of constantly changing average composition. In Table II it is evident, however, that the monostearin is hydrolyzed faster than the tristearin and consequently ought not to accumulate in the system to any extent. We have not yet investigated the behavior of distearin. The possibility of a gross change in the composition of the substrate from tristearin to distearin or to monostearin is definitely ruled out, however, when the nature of the remaining unsaponified fat is known. Instead of containing considerable quantities of mono- or diglycerides, the residual fat found after one-third of the total possible hydrolysis was all tristearin, and was about 90 per cent tristearin after two-thirds hydrolysis.

*Composition of Partly Hydrolyzed Fat*—Tristearin was hydrolyzed by the method previously described, but with an initial amount of 5.04 gm. of tristearin and 10 cc. of pancreas extract in a total volume of 300 cc. When the hydrolysis had reached the desired stage, the liquid remaining (after removal of the samples used to follow the course of the reaction) was made strongly alkaline with potassium carbonate. Kieselguhr was then stirred in and the mixture filtered on a suction funnel through a previously prepared layer of kieselguhr. The filtrate was perfectly clear. The kieselguhr was then washed on the suction funnel with 0.5 per cent  $K_2CO_3$  solution, then with 1 per cent NaCl solution until the washings were neutral, and finally with distilled water. The washed earth was dried in the air and afterward extracted with about 200 cc. of hot absolute alcohol four times. (The residue was then extracted several times with warm ether, but the additional fat recovered was found to be very small.)

The alcoholic solution was evaporated to dryness, and the residue extracted with a large volume of warm ether, filtered to remove calcium stearate, and then shaken with potassium carbonate solution<sup>1</sup> to remove any remaining stearic acid. The ether solution of fat was then evaporated, and left a crystalline residue. The residue was weighed without purification and the melting point<sup>2</sup> and hydroxyl content determined.

The weights of recovered fat agree very well with those calculated from the extent of hydrolysis as determined by the titrations, provided the hydrolysis is regarded as going completely to glycerol. If mono- or distearin were formed instead, the weight of the recovered fat should be about 10 per cent higher, because in that case no glycerol would be lost. We believe the extraction of the kieselguhr was sufficiently complete to permit this reasoning.

Determination of hydroxyl groups was made by the acetylation method of Freed and Wynne (3) with this difference, that the acetylating mixture instead of being boiled was merely warmed gently and then allowed to stand overnight in the absence of moisture. Monostearin (m.p. 81°) was tested by this method with the following results: 53.2 mg. used acetic anhydride equivalent to 2.97 cc. of 0.1 N NaOH and corresponding to 53.2 mg. of monostearin or 100 per cent. Also 50.6 mg. required 2.77 cc. of 0.1 N NaOH, corresponding to 49.6 mg. or 98 per cent monostearin. The tristearin used throughout this work proved to be practically free from hydroxyl groups; 100.4 mg. used acetic anhydride equivalent to 0.07 cc. of 0.1 N NaOH.

*Experiment I*—From a volume of emulsion corresponding to an original weight of 4.45 gm. of tristearin, 2.84 gm. of fat were recovered after 34 per cent hydrolysis had taken place. The recovered fat melted at 71° alone and 70–71° when mixed with an equal weight of tristearin, m.p. 70.5°. On acetylation, 100.0 mg. of fat required acetic anhydride equivalent to –0.02 cc. of 0.1 N NaOH. No hydroxyl was present.

*Experiment II*—From a volume of emulsion corresponding to an original weight of 3.44 gm. of tristearin, 1.13 gm. of fat were recovered after 67 per cent hydrolysis at 40°. Of this 1.01 gm.

<sup>2</sup> Mixed melting points were made by the "remelt" method, after allowing the mixture to exist at least an hour in the solid state.

were recovered by the alcohol extraction (Fraction A), and 0.12 gm. by subsequent extraction with ether (Fraction B). Fraction A melted at 67–68° alone. The mixture with tristearin (m.p. 70.5°) melted at 68–69°. 103.7 mg. of the fat as recovered used acetic anhydride equivalent to 0.51 cc. of 0.1 N NaOH. This corresponds to a mixture of 92.2 per cent tristearin and 8.8 per cent monostearin. A mixture made up of 90 per cent tristearin (m.p. 70.5°) and 10 per cent monostearin (m.p. 81°) was found to melt at 66°. Fraction B melted at 70–71°; mixed with tristearin (m.p. 70.5°), at 70–71°.

Pure tristearin was isolated from Fraction A by recrystallization from ether. 300 mg. of Fraction A yielded 210 mg. of crystals that melted at 70.5°. The crystals mixed with tristearin (m.p. 70.5°) melted at 70–71°. On acetylation, 51.3 mg. of the recrystallized material used acetic anhydride equivalent to 19.83 – 19.85 cc. = –0.02 cc. of 0.1 N NaOH, showing hydroxyl groups absent. Of the residue from the recrystallization of Fraction A, 47.2 mg. used acetic anhydride equivalent to 0.98 cc. of 0.1 N NaOH, corresponding to 17.5 mg. of monostearin or 37 per cent of the residue.

*Hydrolysis of Other Saturated Triglycerides*—Since tristearin may be regarded as outstanding in its resistance to lipolysis, the speed of hydrolysis of an extensive series of the saturated triglycerides has therefore been determined. A part of this work thus repeats in principle the experiments of Terroine (4) and confirms without exception his findings that the highest and the lowest members of the series of saturated triglycerides are hydrolyzed more slowly than are the intermediate members. Since, however, the behavior of tristearin was found to vary greatly with the temperature, the digestion of the entire series was studied at a number of temperatures ranging from freezing to 40°.

The results of these experiments are given in Table III. In all cases the per cent hydrolysis is based on the complete hydrolysis of the triglyceride as a whole. A large number of duplicate determinations and repetitions have been omitted from the data presented, since no significant variations were noted.

A survey of the results shows that the splitting of the fats containing C<sub>8</sub> acids or higher is greatly dependent upon the temperature, the more so the higher the fatty acids. On the other

TABLE III

*Hydrolysis of Several Glycerides at Various Temperatures*

Since in each case one-sixth of 0.00565 mole of substrate was titrated at each determination, the observed increases in titration ( $\Delta$ ), in cc. of 0.1 N KOH, may be calculated from the percent hydrolysis ( $x$ ) by the expressions:  $x = 100 \Delta / 2.83$  for a triglyceride, and  $x = 100 \Delta / 0.94$  for a monoglyceride.

All values are given in per cent.

Substrate	At 0°						At 20°			
	12 hrs.	24 hrs.	48 hrs.	72 hrs.	96 hrs.	144 hrs.	0.5 hr.	1 hr.	2 hrs.	3 hrs.
Triacetin.....	8.3	14.1	17.9	21.1	22.1	24.0	3.8	8.0	12.8	17.3
Tripropionin.....	6.7	15.0	20.8	22.7	24.3	27.5	30.2	33.0	39.8	42.3
Tributylin.....	33.3	52.5	57.0	57.0	60.2	62.0	47.7	54.5	56.5	58.0
Trivalerin.....	34.6	58.3	67.5	73.9	71.6	74.2	41.4	55.7	62.8	65.7
Tricaproin.....	37.1	60.1	70.0	75.5	80.3	86.0	39.7	52.9	71.8	76.5
Triheptylin.....	41.6	66.9	81.6	92.2	96.6	96.9	61.5	69.5	83.3	86.5
Tricaprylin.....	17.9	43.8	76.8	95.4	98.0	98.5	48.4	68.0	81.6	89.0
Tricaprin.....	13.1	25.9	38.1	39.7	53.1	55.7	13.5	23.4	41.6	64.7
Trilaurin.....	3.5	5.8	8.6	15.7	16.6	18.9	9.0	13.8	22.8	33.3
Trimyristin.....	0.3	0.6	2.6	4.5	4.2	5.4	4.2	7.1	11.2	14.1
Tripalmitin.....	0.3	0.6	1.9	2.2	1.9	1.9	0.6	1.3	2.6	2.9
Tristearin.....	0.6	1.6	1.6	3.5	4.5	4.2	0.0	0.6	1.9	2.9

Substrate	At 30°				At 40°			
	0.5 hr.	1 hr.	2 hrs.	3 hrs.	0.5 hr.	1 hr.	2 hrs.	3 hrs.
Triacetin.....			1.37		9.5	16.7	24.2	28.4
Tripropionin.....			37.0		30.4	36.3	43.7	49.4
Tributylin.....	49.6	53.3	56.2		37.2	57.1	58.9	60.8
Trivalerin.....	50.4	60.0	64.5		44.5	60.7	69.5	71.9
Triisovalerin.....	7.2	13.4	22.8	29.1	11.5	18.7	30.6	39.8
Tricaproin.....	64.4	66.4	70.3	73.2	59.0	69.2	72.8	76.0
Triheptylin.....	45.7	65.0	76.1	84.5	61.7	68.2	79.0	82.3
Tricaprylin.....	47.6	72.5	93.5	100.1	61.0	79.6	92.6	96.6
Tricaprin.....	40.7	52.2	66.6	83.4	65.0	80.0	90.1	95.0
Trilaurin.....	31.8	52.9	80.5		66.3	79.6	94.6	99.9
Trimyristin.....	22.3	41.0	65.5		66.3	79.0	91.1	99.2
Tripalmitin.....	10.9	17.9	29.1		27.1	50.0	83.2	97.0
Tristearin.....	6.6	11.9	20.2		32.6	52.2	80.0	87.5
Monostearin.....			36.2					
Monoolein.....	31.7	49.1	66.8					

hand, glycerides of acids lower than  $C_8$  are split little if any faster at  $40^\circ$  than at  $20^\circ$ , and even at  $0^\circ$  their hydrolysis is remarkably rapid, while that of the higher saturated fats is practically nil.

At a low temperature ( $0^\circ$ ) the velocity of hydrolysis increases with the carbon content of the fatty acid up to  $C_7$ , then abruptly decreases. The velocity of splitting is therefore at a maximum with triheptylin. At a temperature sufficient for vigorous action throughout the entire series ( $40^\circ$ ) there appears to be little difference in the rate of splitting observed with glycerides of the acids from  $C_8$  to  $C_{14}$ . Similar findings have just been made by Holwerda, Verkade, and de Willigen (5) and Holwerda (6). Palmitin and stearin, however, split somewhat more slowly. There is thus a plateau of maximum splitting from tricaproilin to trimyristin, instead of the single maximum point as observed at  $0^\circ$  with triheptylin.

At each of the temperatures investigated the lowest glycerides split initially at a greater rate than do the highest ones. The rate soon decreases, however, and the progress of hydrolysis finally becomes very slow. With the lowest members of the series complete hydrolysis must require a very long time, but it is probably obtainable.<sup>3</sup> Tributyrin, for example, was in one experiment hydrolyzed to 74 per cent in 3 days at  $40^\circ$ . On the other hand 3 hours were sufficient to hydrolyze tricaproin almost completely, and triheptylin and all the higher glycerides quite completely. In general, the glycerides containing the  $C_7$  to  $C_{10}$  acids appear to undergo hydrolysis most easily, and the series behaves as if a fatty acid of about this chain length was suited to fit the spacial requirements of the lipase with the least distortion. In this connection it is probably not without significance that olive oil (whose chief constituent is triolein) does not behave like stearin or palmitin at low temperatures, but due perhaps to the  $C_9 = C_9$  structure, acts instead like a saturated glyceride in the  $C_7$  to  $C_{10}$  range.

The favorable effect of increased temperature on the splitting of

<sup>3</sup> While we believe that the effects produced in a few hours are essentially those of pancreatic lipase, the results obtained in these long continued experiments may very well be due to another ferment, such as esterase.

the glycerides of acids from  $C_8$  upward may therefore be related to increased activity which enables the substrate more frequently to "fit" the enzyme. This effect is nevertheless also related to the physical state of the fat. Although the response of the higher fats to increased temperature parallels their increasing melting points, the digestion of tristearin and tripalmitin takes place readily enough at  $40^\circ$ , which is still well under the melting point of either, so the assumption that only liquid or "semiliquid"

TABLE IV  
*Acceleration of Hydrolysis of Tristearin at  $20^\circ$*

Substrate No.*	Hydrolysis			
	30 min.	60 min.	90 min.	120 min.
	per cent	per cent	per cent	per cent
1. Tristearin alone.....	0	0.6		2
2. " + 10% olive oil.....	11	17		22
Maximum hydrolysis possible due to olive oil alone.....	12	12		12
3. Tristearin + 25% olive oil.....	48	61		69
Maximum hydrolysis possible due to olive oil alone.....	30	30		30
4. Tristearin + 500% ether.....	3			6
5. " + 50% cholesterol.....	2		3	
6. " + 50% benzene.....	3		7	9
7. " + 1600% glycerol.....	6		9	

\* The substrate consisted of 504 mg. of tristearin plus the additions noted except in Substrates 2 and 3, where it consisted of 454 mg. of tristearin + 50 mg. of olive oil (Substrate 2) and 378 mg. of tristearin + 125 mg. of olive oil (Substrate 3). In all cases the saponifiable substrate was equivalent to 504 mg. of tristearin. Other details of the experiments correspond to the method as described earlier.

fats may be digested hardly seems justified. On the other hand, the dependence of digestion on the emulsification has often been recognized. It may be demonstrated in the case of tristearin by the addition of other substances known to depress the melting point and presumably to change the character of the surface as well. Thus it was found that the mixture of 10 to 25 per cent of olive oil with the tristearin allows a rapid hydrolysis at  $20^\circ$ , in the course of which tristearin is also decomposed. The observed

splitting was about double what can be accounted for by the complete saponification of the olive oil. Since oleic acid is known to facilitate lipase action, this observation may signify a specific effect, but that assumption seems unreasonable in view of the fact that benzene, ether, and cholesterol, presumably inert substances, act in a similar way though to a far lesser extent (Table IV).

If, however, changes in the physical condition of the fat are alone sufficient to account for the increased activity of tristearin at high temperatures, such an abrupt transition in behavior as occurs when  $C = 8$  is unexpected. This transition seems rather to indicate a difference in the mechanism of the hydrolysis or in the mode of action of the enzyme. Furthermore, the inertness of triisovalerin as compared with trivalerin indicates that details in the constitution of the substrate are critical for the enzymic hydrolysis. As far as physical characteristics are concerned, triisovalerin at  $40^{\circ}$  differs little from its straight chain isomer.

In any case the difference in behavior of the several members of this series of triglycerides at low temperatures is significant. Not only may enzymic lipolysis occur at  $0^{\circ}$  (see also (7)) but it may be surprisingly rapid. In spite of the observation that the splitting of tristearin may be accelerated by the admixture of low melting substances it is also evident that a wide difference will exist between the end-products of lipase action formed at high and at low temperatures. The ease with which the lower fats are hydrolyzed at low temperatures may account for difficulties in preserving fats such as butter. As between tristearin and tributyrin at  $0^{\circ}$ , the difference in reactivity amounts to a variation in the specificity of the enzyme. There are many examples on record of enzymic specificity that may become meaningless unless the temperature of the reaction is stated and considered.

#### SUMMARY

The hydrolysis of tristearin with pancreatic lipase has been carried to practical completion. The course of the reaction is smooth throughout. The hydrolysis of monostearin is approximately monomolecular and goes faster than that of tristearin. The fat remaining after partial hydrolysis of tristearin has been isolated and found to consist almost entirely of tristearin, so the

gross chemical composition of the substrate remains practically unchanged throughout the digestion.

The hydrolysis of the higher, but not of the lower, saturated triglycerides is very dependent on temperature. The difference is so marked that if the behavior at low temperatures only were known, pancreas lipase would be regarded as specific for lower (or for unsaturated) glycerides.

At moderate temperatures, however, a maximum rate of splitting is exhibited by the straight chain saturated glycerides of acids containing approximately 7 to 10 carbon atoms. This does not apply to unsaturated glycerides, for olein behaves as though it contained a  $C_9$  rather than a  $C_{18}$  acid.

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## METABOLISM OF THE BASIC AMINO ACIDS

### I. RATES OF ABSORPTION IN RATS OF THE MONOHYDRO-CHLORIDES OF *d*-LYSINE AND *l*-HISTIDINE

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The importance of a knowledge of the rates at which amino acids pass from the gastrointestinal tract into the blood stream has been emphasized by Wilson and Lewis (1). These authors and Wilson alone (2, 3), Cori (4), Chase and Lewis (5, 6), Sullivan and Hess (7), and Berg and Bauguess (8) have studied the rates of absorption of many of the amino acids and their derivatives by the direct method of Cori (9). We have been interested in the metabolism of the so called "diamino" acids and are reporting absorption rates on lysine and histidine. Work is in progress on the rate of absorption of arginine alone and of each of the three compounds when fed as mixtures. These results will be considered in a later publication.

#### EXPERIMENTAL

The methods used by Wilson and Lewis (1) have been modified only in certain details for this work. White rats, purchased from two local colonies, were employed for these studies. Dog Chow was fed *ad libitum* for at least a week prior to the experiment. All animals were fasted 24 hours previous to administration of the amino acid. The compounds were fed by stomach tube. For this purpose a small, soft rubber catheter was attached to a 2 cc. glass syringe fitted with a metal guard which allowed delivery of a fixed volume (about 2 cc.) of solution to each rat. The quantity of the amino acid administered was determined by measuring the same volume of solution into a 500 cc. volumetric

flask. This was diluted to volume and analyzed for amino nitrogen.

Rats were killed after a period of 1 to 4 hours by a blow on the head. The esophagus and rectum were ligated immediately, and the gastrointestinal tract was removed from the animal, slit open from end to end, and thoroughly washed with about 200 cc. of warm distilled water. To the washings, 5 cc. of 3 per cent acetic acid were added; the material was heated to boiling and filtered by suction on a Buchner funnel. To the filtrate 5 cc. of 20 per cent trichloroacetic acid were added and, after standing, the solution was again filtered. The trichloroacetic acid was largely removed by boiling and the solution was made up to a final volume of 500 cc. The above protein precipitant was considered preferable to tungstic acid for use with the concentrated solutions of the diamino acids.

The procedure was modified in the histidine experiments by adding 5 cc. of 5 per cent trichloroacetic acid and 3 gm. of norit to the washings from the intestines after heat coagulation of the proteins but before filtering off the insoluble residues. A thin layer of norit on the filter paper served as a further filtering aid. This modification gave somewhat lower control values, greatly facilitated the filtration at this point, and eliminated the necessity of a second filtration. Furthermore, the smaller quantity of trichloroacetic acid did not interfere with the subsequent analysis. That there was no loss of the histidine was shown by treating a standard solution of the amino acid by the same procedure. The histidine was recovered completely within the limits of error of the analytical method.

In some of the experiments with lysine a preliminary precipitation with phosphotungstic acid was carried out in the usual manner on a portion of the filtrate. The precipitate was collected and washed with 5 per cent phosphotungstic acid solution. It was treated on the filter with just sufficient 0.5 N sodium hydroxide to bring it into solution and the filtrate and washings were diluted to a volume convenient for analysis.

All solutions were analyzed by the manometric amino nitrogen method of Van Slyke (10). As Van Slyke demonstrated, this procedure gave accurate results even when applied to very dilute solutions. Thus, it was possible to analyze the protein-free fil-

trates without preliminary concentration of the solutions. In a few cases histidine was estimated also by the Koessler and Hanke (11) method as modified by Jorpes (12) with, however, a colorimeter in place of the step photometer. Since these results were in good agreement with those obtained by the Van Slyke method when applied to the same solutions, we have included only the latter data in Tables I and III.

The lysine and histidine used in this work were prepared in our own laboratory and gave all the usual criteria of extreme

TABLE I  
*Amino Nitrogen in Gastrointestinal Tract of Fasting Rats*

Series I (lysine controls)			Series II (lysine controls)			Series III (histidine controls)		
Rat No. and sex	Weight	Total amino N	Rat No. and sex	Weight	Total amino N	Rat No. and sex	Weight	Total amino N
	gm.	mg. per 100 gm.		gm.	mg. per 100 gm.		gm.	mg. per 100 gm.
1 M.	196	7.0	25 M.	299	2.2	48 M.	240	2.59
4 F.	154	7.3	26 "	273	2.7	49 "	249	1.70
9 "	177	7.4	29 "	255	4.7	50 "	269	3.33
13 M.	225	15.0	30 "	293	4.5	57 "	264	2.07
20 F.	164	10.1	37 "	294	2.9	60 "	303	2.47
21 M.	259	10.8	38 "	332	4.0	61 "	217	2.15
			40 "	275	6.1	71 "	177	2.36
						79 "	180	1.96
						88 "	151	2.24
						91 "	138	3.40
Average.....		9.6			3.9			2.43

purity. The compounds were fed and calculated in all cases as the monohydrochlorides.

*Amino Nitrogen in Gastrointestinal Tract of Control Rats*—The animals used for the lysine studies were secured from two sources and a series of controls was established for each group. These values are included in Table I. The residual amino nitrogen for Series I was considerably higher and more variable than in Series II. Wilson and Lewis (1) found similar variations with the season of the year, but, since all of our experiments were performed within a period of less than 2 months, and, since all of our animals were maintained on the same stock ration for some time

prior to study, we can only suggest that this variability is related to the source of the rats.

With the slight modifications introduced in the histidine experiments it became necessary to secure a third series of control values. The animals were from the same source as those of Series II. It should be pointed out that, probably owing to the use of norit, the average control value in this series is somewhat lower than in Series II and the individual variations are less marked. The treatment applied to these solutions rendered them water-clear, whereas those solutions analyzed in the lysine experiments were sometimes slightly turbid. It appears possible that very finely divided protein suspensions were more completely removed when the norit was employed.

*Lysine Absorption*—The data are given in Table II. Series I of Table I presents control values for data recorded in Table II for all rats numbered up to and including 24. Series II of Table I serves as control values for Rats 27 to 47 inclusive. There were no significant differences between the rates obtained with or without the use of phosphotungstic acid.

The most striking observation made is the slight amount of absorption occurring after the 2 hour period and, particularly, the almost complete cessation of absorption during the 4th hour although a large residue of lysine is always present in the intestine. This phenomenon is somewhat similar to that observed by Wilson and Lewis (1) in the case of *d*-glutamic acid fed as the monosodium salt. These workers observed a possible decrease in rate for the 2nd hour and a very decided drop in absorption rate during the 3rd hour with, however, an apparent increase in rate the 4th hour. These authors discount the likelihood of the delayed increase but feel justified in believing that there is "a greater initial rate than is found later." This conclusion for glutamic acid is entirely in line with our findings upon lysine. The actual quantities of lysine absorbed are, however, much lower for the corresponding periods than was observed with the dicarboxylic acid (29.2 and 21.9 mg. of free lysine during the 2 and 3 hour periods as compared with 85.8 and 60.1 mg. of free glutamic acid). When computed on the basis of nitrogen content the figures for lysine are 5.72 mg. and 4.22 mg. for the 2 and 3 hour periods respectively. From the data of Wilson and Lewis (1) the corresponding values for glutamic acid would be 8.18 mg. and 5.72 mg.

TABLE II  
Rate of Absorption of *d*-Lysine Monohydrochloride

Rat No. and sex	Weight	Absorption time	Amount fed	Amount recovered determined by		Absorption rate calculated by	
				Method A*	Method B†	Method A*	Method B†
	gm.	hrs.	mg. per 100 gm.	mg. per 100 gm.	mg. per 100 gm.	mg. per 100 gm. per hr.	mg. per 100 gm. per hr.
5 M.	197	2	238	157		40.5	
8 F.	153	2	307	220		43.5	
11 "	151	2	311	245		33.0	
17 M.	222	2	215	120		47.5	
18 "	165	2	289	185		52.0	
36 "	231	2	224	172	160	26.0	32.0
41 "	254	2	213	146	136	33.5	38.5
44 "	277	2	195	141	133	27.0	31.0
47 "	301	2	179	112	105	33.5	37.0
Average.....						37.3	34.8
6 F.	138	3	340	225		38.3	
14 M.	163	3	293	200		31.0	
15 "	163	3	293	217		25.3	
16 "	165	3	289	192		32.3	
22 "	152	3	311	236		25.0	
27 "	308	3	153	92		20.3	
32 "	196	3	263	175	138	29.3	41.7
39 "	293	3	176	111	90	21.7	28.7
42 "	310	3	174	107	100	22.3	24.7
45 "	282	3	192	105	93	29.0	33.0
Average.....						27.5	32.0
10 M.	161	4	291	198		23.2	
23 F.	160	4	295	223		18.0	
24 "	152	4	311	231		20.0	
28 M.	307	4	154	66		22.0	
31 "	277	4	187	116	98	17.8	22.2
33 "	297	4	174	104	80	17.5	23.5
35 "	282	4	183	106	86	19.2	24.2
43 "	200	4	270	155	141	28.7	32.2
46 "	333	4	162	71	69	22.8	23.2
Average.....						21.0	25.1

\* Calculated from total amino nitrogen content of intestinal washings.

† Calculated from amino acid nitrogen precipitated by phosphotungstic acid.

Of the amino acids thus far studied only cystine (2) exhibits an absorption rate as low as that of lysine when expressed as mg. per 100 gm. of rat per hour. If calculated upon the amino nitrogen content, lysine is absorbed at about the same rate as tryptophane (8), valine, and the isomeric leucines (6) but at a definitely lower rate than alanine and glycine (1, 3).

*Histidine Absorption*—It is apparent from a comparison of Tables II and III that histidine is absorbed more rapidly than lysine when the two are calculated on the basis of weight. When computed as total amino nitrogen absorbed, the rates for the two

TABLE III  
*Rate of Absorption of l-Histidine Monohydrochloride*

Rat No. and sex	Weight	Absorption time	Amount fed	Amount recovered	Absorp- tion rate	Rat No. and sex	Weight	Absorption time	Amount fed	Amount recovered	Absorp- tion rate
	gm.	hrs.	mg. per 100 gm.	mg. per 100 gm.	mg. per 100 gm. per hr.		gm.	hrs.	mg. per 100 gm.	mg. per 100 gm.	mg. per 100 gm. per hr.
58 M.	240	1	169	90	79	53 M.	281	2	143	16	63.5
63 "	258	1	157	79	78	55 "	287	2	141	40	50.5
64 "	312	1	130	45	85	62 "	266	2	152	42	55.0
72 "	238	1	200	98	102	70 "	248	2	191	41	75.0
74 "	229	1	208	92	116	73 "	215	2	221	61	80.0
76 "	189	1	251	157	94	75 "	221	2	215	70	72.5
86 "	159	1	229	101	128	77 "	204	2	233	90	71.5
						85 "	147	2	248	84	82.0
						87 "	143	2	255	78	88.5
Average .....					97.4						71.0

compounds are quite similar (5.19 and 5.72 mg. for histidine and lysine respectively). Owing to the lower solubility of the histidine monohydrochloride and its rather rapid absorption this acid was studied for only 1 and 2 hour periods. Considerable variation in individual cases may be observed. This was to be expected, of course, since the experimental periods were short and since the factor for conversion of amino nitrogen to histidine monohydrochloride is large. Thus a variation of 1 mg. in the control value for amino nitrogen introduces an error of 13.7 mg. in the result for the 1 hour period.

Again, with histidine there seems to be a distinctly lower rate of absorption during the 2nd hour but this might be explained by the fact that an average of at least half of the total histidine administered was taken up in the 1st hour, thereby leaving an insufficient amount to maintain a maximum absorption rate during the 2nd hour. In terms of free acid, the absorption rates for the 1 and 2 hour periods respectively are 79 and 57 mg. per 100 gm. of rat per hour. While it is difficult to compare these results with some of the others reported for different amino acids, it appears in general that histidine disappears from the intestinal tract at about the same rate as tryptophane (8) and probably somewhat slower than glycine and alanine (1, 3).

An attempt to correlate the absorption rates of the various amino acids thus far studied with their known properties scarcely seems justified at this time. It might be pointed out, however, that lysine which in rats displays a slow absorption rate, is metabolized slowly in dogs following its administration by intravenous injection (13). In the latter instance a very large fraction of the acid is excreted in the urine, while the blood urea nitrogen level is not changed appreciably. Glycine, on the contrary, when given intravenously, gives rise to a greatly increased blood urea nitrogen level and a relatively much lower excretion of amino nitrogen in the urine. Whether or not there is a general relationship between the rate of absorption and the rate of metabolism of the other amino acids remains to be demonstrated.

#### SUMMARY

The average absorption rates of lysine monohydrochloride as determined by Cori's method were 37.3, 27.5, and 21 mg. per 100 gm. of rat per hour for the 2, 3, and 4 hour periods respectively. The rates for histidine monohydrochloride for the 1 and 2 hour periods were 97.4 and 71 mg.

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## 146      Metabolism of Basic Amino Acids.    I

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## THE REGENERATION OF BLOOD LIPIDS FOLLOWING A SINGLE MASSIVE HEMORRHAGE IN RABBITS

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While lipids are generally believed to pass continuously from plasma to tissues and *vice versa*, very little is actually known about the rate at which this exchange takes place. Under conditions of normal health, the concentration of lipids in plasma remains fairly constant (2) and this, together with the fact that lipids are not water-soluble and hence cannot diffuse like crystalloids across an animal membrane, has suggested that the turnover of lipids in plasma is probably not a rapid one. No measurement has been made of the absolute extent of this exchange and no satisfactory procedure appears to have been devised for doing so.

The object of the present study was to compare the relative rates at which the several plasma lipids are replaced following removal of a considerable amount of lipid by a single, large hemorrhage. Although this procedure is an obvious method of investigating the problem, it does not appear to have been previously systematically studied. For the most part, the only data available in this connection are incidental observations made by those who have used repeated hemorrhage to produce an experimental anemia in animals. For example, Chamberlain and Corlett (5) found that plasma cholesterol dropped from a value of 37 to 23 mg. per cent during the removal of 57 cc. of blood from a rabbit.

Other relevant data appear in the reports of those who have determined the time required for identifiable fats to appear in blood after they have been given by mouth. Thus Sinclair (7) found that elaidic acid appeared in significant amounts in plasma phospholipids not earlier than 8 hours after it had been given by mouth to cats. Experiments of this type do not have a direct

bearing on the question, because lipids in the bowel are really outside the body and cannot be considered in the same light as lipids in the tissues.

The procedure adopted was to remove a considerable amount of blood from a rabbit and compare the change in lipid values of blood plasma and red blood cells over an interval varying from 3 to 48 hours. While the removal of blood means the simultaneous removal of lipids in blood, the latter cannot be considered as a static quantity. During bleeding some lipids may be added to blood by certain tissues and removed by others. When the lipid composition of blood is determined at stated intervals after bleeding, the difference in concentration from the initial values does not necessarily indicate the amount of lipid added to blood in that interval, since the amount of any lipid leaving blood after bleeding and during this same interval is not estimated. What was determined was merely the effect of a single bleeding on the concentration of blood lipids, the concentration being the net result of the exchange of lipids between blood and tissues at the time a sample of blood was removed for analysis.

The demonstration that one lipid of blood is restored to its initial concentration in a shorter time than another would not necessarily mean that that lipid was added to blood at a faster rate than others, because it could be argued that the reason for a more rapid return to initial values was perhaps a slower rate of removal from plasma by some tissues than addition by others. These considerations need to be borne in mind when the data described below are interpreted.

Not more than two samples of blood could be taken from one animal without complicating the experimental plan. Repeated hemorrhage in rabbits produces a lipemia (1) which is apparently due to a deficiency of oxygen (8). While the lipemic effect of hemorrhage in rabbits could not be eliminated entirely even after a single bleeding, it could be and was minimized by taking only two samples of blood from one animal and using separate animals to study the various intervals.

A colony of thirty male rabbits was divided into five groups of six animals each. Approximately 30 cc. of blood, representing about one-quarter of the blood volume of a rabbit, were removed from the marginal ear veins, previously made hyperemic by rub-

bing. No anesthetic agent was used, since this in itself may affect blood lipid values (3). Second samples of at least 15 cc. of blood were taken from one group of animals 3 hours later and from the other groups 6, 12, 24, and 48 hours later respectively. There were four casualties from the initial bleeding, leaving two groups with but five animals each and one group (at 48 hours) with only four animals for the second sample of blood. No attempt was made to remove food from the cages of these animals, since Horuchi (6) has shown that a postprandial lipemia does not occur

TABLE I

*Changes in Lipid Content of Rabbit Plasma at Varying Periods Following Massive Hemorrhage*

All means represent per cent of the initial value before hemorrhage.

Time after bleeding hrs.	Value	Hemo-globin	Total lipid	Composition of total lipid					
				Neu-tral fat	Total fatty acids	Cholesterol			Phos-pho-lipid
						Total	Ester	Free	
3	Mean	-25	-14	+18	-4	-37	-39	-26	-39
	Standard deviation	10	10	16	11	8	14	13	19
6	Mean	-26	-4	+21	+1	-30	-40	-10	-20
	Standard deviation	4	26	51	31	19	20	41	19
12	Mean	-26	-7	-15	-10	+15	+7	+16	-6
	Standard deviation	4	8	8	11	11	17	20	25
24	Mean	-29	+1	+8	+4	-6	-7	+12	-1
	Standard deviation	6	15	14	16	11	33	31	27
48	Mean	-26	+34	+88	+45	+9	+5	+14	+13
	Standard deviation	10	28	45	38	36	32	30	27

in rabbits normally or after a single bleeding, and this has been repeatedly confirmed (1).

Blood was collected in flasks containing 1 to 2 mg. of heparin (Connaught Laboratories) per 10 cc. of blood. Boyd and Murray (4) have shown that heparin has not the undesirable effect of anti-coagulant salts in altering the apparent distribution of lipids between plasma and red blood cells. From the centrifuged blood, extracts of plasma and of the red blood cells were made by cold dilution in alcohol-ether (4). The filtered extracts were analyzed by a modification of the oxidative micromethods of Bloor as used in previous studies.

The percentage change from the initial values was calculated for each lipid in each animal during the interval which elapsed from the time of bleeding. The number of red blood cells and the percentage of hemoglobin were also determined on each sample of blood and the results agreed in demonstrating that the initial bleeding removed about 25 per cent of the original blood present in the entire animal and that no appreciable regeneration of red blood cells occurred as late as 48 hours afterwards. Mean percentage changes have been summarized in Table I. To show the variation in results and for purposes of determining the statistical significance of the means, their standard deviations have also been calculated and included in Table I.

At no time did significant changes take place in the lipid content of the red blood cells. Hence it would be superfluous to report in detail the means and standard deviations of the minor differences noted in the same manner as those of plasma recorded in Table I.

Immediately after bleeding there occurred a considerable fall in the *phospholipid* content of plasma. After an interval of 3 hours, which was about the minimum found necessary to permit the animals to recover from the shock of the initial hemorrhage, the average decrease in plasma phospholipid was 39 per cent. The greatest loss after 3 hours was 68 per cent, the least 10 per cent; a loss occurred in all of the animals and since the standard deviation was less than one-half of the mean (Table I), the change was statistically significant. Between 3 and 6 hours after bleeding, the concentration of phospholipid rose considerably; at 6 hours the mean loss was 20 per cent which, considered by itself, was not statistically significant. Between 12 and 24 hours the mean values of plasma phospholipid reached the initial mean and at 48 hours had exceeded the initial mean by 13 per cent. This late lipemic effect occurred in but one-half of the animals and was not significant.

The mean decrease in the plasma phospholipid was greater at 3 hours than the mean loss of blood as estimated by the dilution of hemoglobin (Table I). This may be due to one of two causes. First, it may simply represent the normal variation in the method, or, secondly, it may mean that after hemorrhage the mechanism

responsible for the addition of phospholipid to plasma cannot keep pace with the rate at which other tissues remove it.

Changes in plasma *cholesterol ester* content were found similar to the changes in phospholipid. As seen in Table I, there was an average decrease of 39 to 40 per cent at 3 to 6 hours after bleeding; this decrease occurred in over 80 per cent of the animals and was statistically significant. 12 hours after hemorrhage the concentration of cholesterol esters in plasma had returned to the initial level and there was no evidence of a lipemic tendency up to 48 hours. Like phospholipid, the initial loss of cholesterol esters was greater on the average than the initial loss of blood.

Variations in the concentration of *free cholesterol* were similar to those noted for phospholipid and cholesterol esters. The mean values for free cholesterol did not fall as low and rose more quickly than did corresponding values for phospholipid and cholesterol esters. Also after an interval of 12 hours the mean values for free cholesterol were 13 to 16 per cent above the initial values, though these latter increases were not statistically significant.

These results demonstrate that following the removal of one-quarter of the blood of rabbits, 12 hours are required for the return of the normal equilibrium between the concentration of phospholipid and cholesterol esters of plasma and of the tissues. During this interval phospholipid and cholesterol esters are added to plasma at a faster rate than they are removed, with the possible exception that in the first 3 to 6 hours the rate of removal exceeds the rate of addition. Changes in plasma-free cholesterol values are similar but the equilibrium of free cholesterol may possibly be restored more rapidly.

The effect of hemorrhage on plasma *neutral fat* values was entirely different. At no time was there a statistically significant change from the initial values in the concentration of this substance. The mean values (Table I) were elevated at all intervals studied following bleeding except at 12 hours when the mean was found below the initial level. At 48 hours there was a marked increase of 88 per cent in the average neutral fat content of plasma, which was evidence of an impending lipemia (1).

These results clearly demonstrate that neutral fat readily passes into plasma from the tissues. They do not necessarily show that

neutral fat enters plasma at a faster rate than other lipids, since it may be that neutral fat is not removed from plasma as quickly as are other lipids. The permissible conclusion is that following hemorrhage the concentration of neutral fat in plasma is reestablished much earlier than that of other lipids.

#### SUMMARY

A differential lipid analysis was performed by oxidative micro-methods on the plasma and red blood cells of thirty rabbits before and at an interval of from 3 to 48 hours after removal of one-quarter of the blood of the animal. 3 hours after the hemorrhage the plasma content of phospholipid and cholesterol esters was decreased by 40 per cent and initial values were not reached for 12 to 24 hours. The concentration of plasma-free cholesterol decreased to a somewhat lesser extent and reached the initial values somewhat earlier. Plasma neutral fat values varied considerably but at no time was there a significant decrease. There were no significant changes whatsoever in the lipid content of the red blood cells.

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## STUDIES ON THE CHEMISTRY OF BLOOD COAGULATION

### VI. STUDIES ON THE ACTION OF HEPARIN AND OTHER ANTI- COAGULANTS. THE INFLUENCE OF PROTAMINE ON THE ANTICOAGULANT EFFECT IN VIVO\*

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Heparin, which was discovered in liver by Howell and Holt (1), is according to recent work (2) considered as a mucoitin polysulfuric acid. A number of highly potent synthetic anticoagulants which consist of sulfuric acid esters of polysaccharides and other high molecular alcohols have been described by Bergström (3) and by Chargaff, Bancroft, and Stanley-Brown (4). The latter authors also have reported a method for the determination of the potency of anticoagulants (5).

While the proper physiological function of heparin in the body is not yet established, it is undoubtedly the most potent anticoagulant known. By the work of Charles and Scott (6) heparin has become available in sufficient purity and quantity to make its use in physiological and clinical experiments feasible. In recent times there have been attempts to use heparin for the prevention of thrombosis, for the preservation of blood in transfusions, etc. (7-11).

In the present paper a study is presented of the mode of action of various anticoagulants in dogs. The substances used were (1) heparin from beef lungs, (2) sodium salt of cellulose disulfuric acid  $(C_6H_8O_{11}S_2Na_2)_x$  (4), and (3) potassium salt of polyvinyl sulfuric acid  $(C_2H_3O_4SK)_x$  (4).

As was to be expected, different anticoagulants, when applied

\* Study of the mechanism of thrombosis and embolism supported by the Carnegie Corporation of New York.



in doses containing the same amount of inhibitor units (5), exerted a quantitatively different effect. In the case of heparin, the animal organism seems able to dispose of it in a comparatively short time. Whether this is brought about by rapid combination of the heparin with the proteins of the tissues or of blood, by its excretion (*cf.* (12)), or by its destruction, it not yet established. We have attempted to protract the short lived activity of heparin by combination with a protamine, *viz.* salmine. This was done in view of the promising results obtained with the combination of insulin and protamine (13). In the case of heparin the effect was unexpected: the anticoagulant action of heparin *in vivo* was entirely stopped by protamine. The significance of these findings and some possible practical applications will be discussed later in this paper.

#### EXPERIMENTAL

##### *Material*

*Heparin*—The heparin used was a highly purified preparation from beef lungs, which contained between 600 and 1000 inhibitor units (5) per mg. The sterile solutions were so adjusted that 1 cc. contained 10,000 inhibitor units. This material was placed at our disposal by Hoffmann-La Roche, Inc., Basel and Nutley, New Jersey.

*Sodium Salt of Cellulose Disulfuric Acid*—This substance, the preparation of which has been described previously (4), formed a white, water-soluble powder containing 250 inhibitor units per mg. The solution in physiological saline used had a concentration of 5000 inhibitor units per cc. and was sterilized at 15 pounds pressure for 20 minutes.

*Potassium Salt of Polyvinyl Sulfuric Acid*—This substance has likewise been described before (4). It contained 250 inhibitor units per mg. The strength of the sterile solution of the compound in saline was similar to that of cellulose sulfuric acid.

*Salmine*—This protamine, which was used in the form of its sulfate, was prepared according to Scott and Fisher (14). The preparations were placed at our disposal by E. R. Squibb and Sons, New Brunswick, New Jersey, and by Eli Lilly and Company, Indianapolis, Indiana. Salmine was used as a 2.2 per cent solution in physiological saline and sterilized by heating to a pressure of 15 pounds for 20 minutes.

### Methods

*Determination of Anticoagulant Activity*—The potency of the various anticoagulants used was determined by the method previously described (5), chicken plasma being employed as the substrate. The same technique also was applied for the study of the influence of protamines on anticoagulant effects *in vitro*.

*Administration of Anticoagulants*—The dogs used in the experiments were healthy animals, weighing between 10 and 16 kilos. They received the routine kennel diet. The experiments with anticoagulants were carried out after a fasting period of about 20 hours. Unless otherwise stated, the anticoagulants were injected intravenously. The recurrent tarsal veins were used both for the injection of substances and the withdrawal of blood, except in a few cases in which the jugular vein had to be used. It was not necessary to apply an anesthetic. For the determination of clotting time, 4 to 5 cc. of blood were withdrawn into 5 cc. syringes, 20 gage needles being used. In withdrawing blood, care was taken to damage the surrounding tissue as little as possible.

*Determination of Blood Clotting Time*—For the serial determinations a very simple method was found practicable. The blood (1.5 to 2 cc.) was transferred into a small Pyrex test-tube (10 × 75 mm.). The tube was covered with a strip of waxed paper and inverted at intervals of 30 seconds until a clot had formed. The majority of normal clotting times observed lay between 3 and 5 minutes.

*Determination of Plasma Clotting Time*—To 3.5 cc. of blood 0.075 cc. of 0.1 N sodium oxalate was added. The mixture was centrifuged for 5 minutes, and the supernatant plasma was drawn off and tested by mixing 0.1 cc. of plasma with 0.1, 0.2, and 0.3 cc. respectively of a 0.025 N calcium chloride solution. The determination was carried out at 40°. The normal plasma clotting times observed were between 1½ and 2 minutes. The amount of trauma suffered by the vein, when blood was withdrawn, appeared to influence these values much more than it did the whole blood clotting times.

*Hematocrit, Hemoglobin, Red Blood Cell, and Platelet Counts*—These determinations were carried out in part of the experiments, oxalated blood being used. The methods used were the routine laboratory procedures.

TABLE I  
Effect of Intravenous Injection of Heparin on Blood Clotting Time

Time after in- jection	2500 units per kilo				5000 units per kilo				7000 units per kilo				10,000 units per kilo				15,000 units per kilo							
	Experi- ment 1	min. sec.	Experi- ment 2	min. sec.	Experi- ment 3	min. sec.	Experi- ment 4	min. sec.	Experi- ment 5	min. sec.	Experi- ment 6	min. sec.	Experi- ment 7	min. sec.	Experi- ment 8	min. sec.	Experi- ment 9	min. sec.	Experi- ment 10	min. sec.	Experi- ment 11	min. sec.	Experi- ment 12	min. sec.
Start	1 35	1 45			3 00	4 30	4 30	4 30	4 30	2 00	1 30	5 45	1 30	5 45	4 30	1 45	4 30	1 45	2 15	1 45				
10										>180	>180	>180	>180	>180	>180									
20																								
30			1 30		>180	>180	>180	>180	>180	>180	>180	>180	>180	>180	>180									>180
40	4 30																							
60					>180	>180	>180	>180	>180	>180	>180	>180	>180	>180	>180									
90					19 00	12 00	12 00	60 00	60 00	18 00									>180					
100																								
120	1 30	1 15			8 00	5 00	5 00	10 30	10 30	13 00			50 00	50 00										
150																								
160																								
170																								
180					8 00								25 00	25 00	12 00				>180					25 00
210																								
220																								
240	1 00	1 00								4 30	4 30	4 30	10 00	10 00	4 30									8 00
280																								
300					5 45								10 00	10 00	2 00							25 00	3 00	
320																						17 00		
390																								
430															1 35				6 30		1 30			3 00

TABLE II  
*Effect of Intravenous Injection of Heparin on Plasma Clotting Time*

Time after injection	2500 units per kilo			5000 units per kilo			7000 units per kilo			10,000 units per kilo			15,000 units per kilo		
	Experiment 1	Experiment 2		Experiment 3	Experiment 4	Experiment 5	Experiment 6	Experiment 7	Experiment 8	Experiment 9	Experiment 10	Experiment 11	Experiment 12		
	min. sec.	min. sec.		min. sec.	min. sec.	min. sec.	min. sec.	min. sec.	min. sec.	min. sec.	min. sec.	min. sec.	min. sec.		
Start															
10															
20															
30		2 15													
40	1 30														
60				6 00			54 00								
90				6 10	2 20	2 30	45 00			15 00			8 00		
100							5 00								
120	1 30	2 00		3 45	2 00		11 00								
150								3 10		4 45					
160						1 20				3 15					
170															
180				2 25				1 30	4 00		6 00		3 50		
210					2 00	1 30				1 35		4 00			
220							2 50								
240		0 50						1 35	4 00		6 00				
280					2 25							2 45			
300			1 20					1 30	2 10		3 00	2 45	3 10		
320												2 00			
390											1 20				
430									1 15						

*Experiments with Heparin*

The results obtained in a series of experiments with heparin, with amounts of from 2500 to 15,000 inhibitor units per kilo, are summarized in Tables I and II. It will be seen that the smallest effective dose was found at 5000 units per kilo. The intravenous injection of smaller amounts gave inconclusive results (Experiments 1 and 2). If the prolongation of the blood clotting time to 4 times its normal value is considered as the minimum anti-coagulant effect, and the fluidity of the blood for more than 3

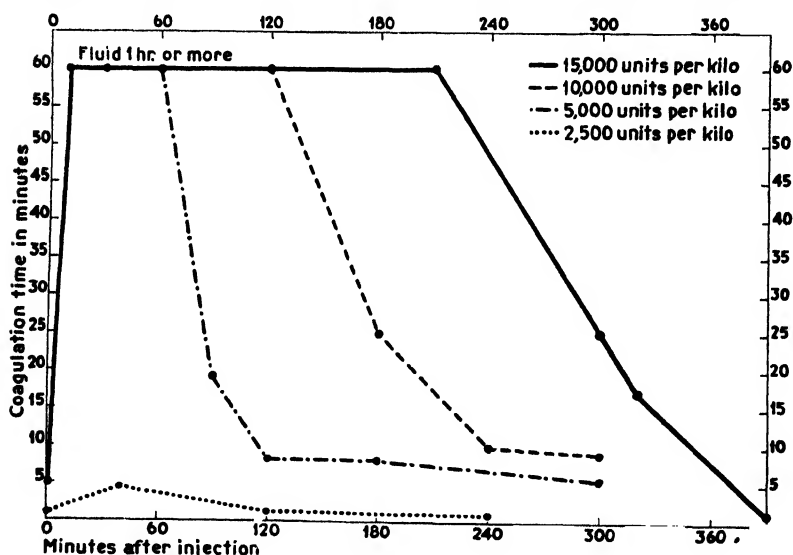


FIG. 1. Effect of varying doses of heparin on the blood clotting time

hours as the maximum effect to be measured, it can be said that while this maximum effect is reached very rapidly with all dosages used, the duration of a discernible anticoagulant action is dependent upon the amount of heparin used. This is shown in Fig. 1, in which the time elapsed after the injection of varying amounts of heparin is plotted against the clotting times observed. With large doses the minimum anticoagulant effect was demonstrable for about 5 hours.

From a comparison of the values for the plasma clotting times given in Table II with those for whole blood given in Table I it

will be apparent that there is a consistent discrepancy between these two sets of figures. The plasma clotting values not only have the tendency to return to the normal level much more rapidly than do the blood clotting values; even their maximum response is comparatively weak. One of the possible explanations for this phenomenon was that the injected heparin soon became adsorbed on the blood cells and exerted its activity in the adsorbed state. It then could be readily understood that, as soon as the blood cells were removed from the oxalated plasma in the plasma clotting test, the anticoagulant effect was destroyed. This assumption, however, seems to be disproved by the experi-

TABLE III

*Comparison of Clotting Times of Blood, Plasma, and Recalcified Oxalated Plasma after Injection of 10,000 Units of Heparin per Kilo*

Time after injection	Clotting time			
	Blood		Plasma	Recalcified oxalated plasma
	(1)		(2)	(3)
min.	min.	sec.	min.	min. sec.
Start	1	30		1 45
10	>240		>120	>120
60	>240		>120	>120
120	50		>180	3 10
180	25		>60	1 30
240	10		11	1 35
300	10		10	1 30

ment reproduced in Table III, in which the clotting times after injection of 10,000 inhibitor units per kilo are compared for (1) whole blood, (2) plasma, obtained by centrifugation without addition of sodium oxalate, (3) recalcified oxalated plasma. Column 2 is similar rather to Column 1 than to Column 3. Whatever the explanation may be, it is obvious that for the testing of heparin *in vivo* the blood clotting time is more sensitive than the plasma clotting time.

#### *Experiments with Synthetic Anticoagulants*

A selection of experiments conducted with cellulose sulfuric and polyvinyl sulfuric acids is given in Table IV. Considerably

## 160      Chemistry of Blood Coagulation. VI

smaller doses than in the case of heparin sufficed to produce the maximum effect. Particularly, the polyvinyl sulfuric acid exerted an extremely strong anticoagulant effect *in vivo*. This may be due to the fact that the body is not able to dispose of these unphysiological compounds as effectively and rapidly as in the case of heparin. The possible practical applications of polyvinyl sulfuric acid are under investigation at present.

TABLE IV  
*Effect of Intravenous Injection of Synthetic Anticoagulants on Blood Clotting Time*

Time after injection	Clotting time			
	Cellulose sulfuric acid		Polyvinyl sulfuric acid	
	Experiment 13	Experiment 14	Experiment 15	Experiment 16
min.	min. sec.	min. sec.	min. sec.	min. sec.
Start	3 00	3 30	3 30	3 30
10				>180
20	35 00			
30		>60	>120	
60	25 00			
80		>60	>120	
100				>180
130			>120	
140		17		
180	5 15			>180
220		4 30		>180
270			45	
320			5	36
360	5			25
480				12

In Experiments 13, 15, and 16 2500 inhibitor units per kilo, in Experiment 14 5000 inhibitor units per kilo were injected.

*Influence of Protamine on Activity of Anticoagulants*

The experiments with salmine were started in view of the stabilizing action of protamines on insulin (13). According to Waldschmidt-Leitz and collaborators (15), protamines have an inhibiting effect on the clotting of blood *in vitro*. We have been able to confirm this finding. However, with the amounts of salmine used in our animal experiments no anticoagulant action was

observed. Several experiments, in which 11 mg. per kilo of salmine were injected intravenously, failed to show any deviation from the normal clotting values.

It was found that the activity of heparin could be entirely stopped by intravenous injection of protamine. If salmine is applied intravenously immediately before or after the intravenous injection of heparin, there is no anticoagulant effect. The precipitate obtained, when salmine and heparin are mixed, is equally ineffective. If the application of heparin is followed after some

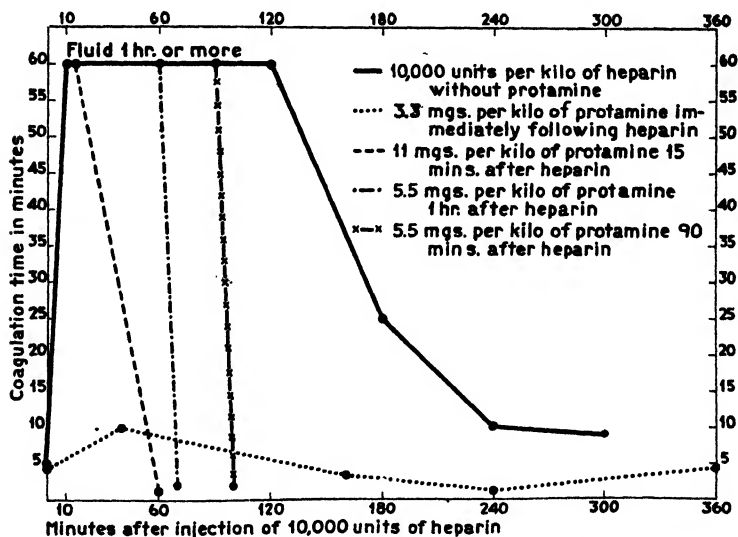


FIG. 2. Influence of salmine on the activity of heparin (10,000 units per kilo).

time by the injection of salmine, the prolonged clotting time produced by heparin is immediately brought back to the usual level. Some instances of the action of protamine are shown graphically in Fig. 2. In more than twenty experiments in which varying amounts of both heparin and salmine were used, only one slightly divergent result was observed. In Table V some of these experiments are summarized. The smallest amount of salmine necessary for this "antiheparin" effect was determined in a series of experiments, a few of which are reproduced in Fig. 2. The mini-



## 162      Chemistry of Blood Coagulation.    VI

imum dose of salmine sufficient for the inactivation of 10,000 inhibitor units seems to be in the neighborhood of 3 mg. The values obtained for the plasma clotting time are omitted from the tables. They followed the blood clotting times quite closely. The subcutaneous injection of protamine proved ineffective.

TABLE V  
*Influence of Protamine on Activity of Heparin*

Time after injection of heparin	Clotting time of dog blood						
	5000 heparin units per kilo			10,000 heparin units per kilo			
	Experi- ment 17	Experi- ment 18	Experi- ment 19	Experi- ment 20	Experi- ment 21	Experi- ment 22	Experi- ment 23
min.	min. sec.	min. sec.	min. sec.	min. sec.	min. sec.	min. sec.	min. sec.
Start	3 ←	2 25	2	4 30←	3	7 30	2 30
10				6 30			
30	2	>180 ←	>180 ←				
40		8	10	10			
60	3 30				>180 ←	>180 ←	
70					2	3 30	
80		6					
90							>180 ←
100			9				1 30
120	5 30				5 15	4 30	
180		5	6 30	3			
210	5						4
240				1 30			
300		4	6		5	4 30	2 30
360				4 30	4 30	6	2 45
430					4 30	2	
515					5	4	
1080	4 30						
1440					4 30	2 30	

Salmine injected: Experiment 20, 3.3 mg. per kilo; Experiments 18, 19, 21, 23, 5.5 mg. per kilo; Experiments 17, 22, 11.0 mg. per kilo. The arrows indicate the last reading before the administration of protamine.

The anticoagulant effect produced by cellulose sulfuric acid is also prevented by protamine, as shown in Table VI. With polyvinyl sulfuric acid the results have not yet been as conclusive. We have observed cases in which protamine had no effect on the anticoagulant action of this compound. This question is being studied at present.

In view of the interesting results obtained with salmine in animals, coagulation experiments were carried out with this pro-

TABLE VI

*Influence of Protamine on Activity of Cellulose Sulfuric Acid*

Time after injection of anticoagulant	Clotting time of dog blood				
	2500 inhibitor units per kilo				5000 inhibitor units per kilo
	Experiment 24		Experiment 25		Experiment 26
min.	min.	sec.	min.	sec.	min. sec.
Start	2		3		3
20	>180	←	60	←	>180 ←
30			4	30	6
60	12				
140			6	30	3
180	5				
240			4	30	7
360	2	45			
420					4 30

Salmine injected: Experiments 24, 25, 5.5 mg. per kilo; Experiment 26, 16.5 mg. per kilo. The arrows indicate the last reading before the administration of protamine.

TABLE VII

*Inactivation of Heparin by Salmine*

Each tube contains 48 inhibitor units of heparin in 0.1 cc. of activated chicken plasma.

Salmine	Clotting time
mg.	min.
0	>200
0.005	>200
0.01	>200
0.02	27
0.04	27
0.08	27

tamine, chicken plasma being used as substrate according to the method described previously (5). Salmine itself was found to contain about 10 inhibitor units per mg. The effect of the addi-

tion of protamine to plasma containing an inhibitor was as manifest as in the injection experiments. The following anticoagulants were found to be completely or almost completely inactivated: heparin, cellulose sulfuric acid, polyvinyl sulfuric acid. The lipid inhibitors from brain and blood cells, which have recently been isolated (16), and two new synthetic anticoagulants of lipid nature, cerebrin sulfuric acid and kersin sulfuric acid (17), were likewise entirely inactivated. There was a marked, but less distinct influence on hirudin. On the other hand, the anticoagulant action of sodium oxalate and sodium citrate was not affected. This is in harmony with the view that the inactivation of high molecular anticoagulants by protamine is due to the formation of insoluble complexes which remove the anticoagulants from the blood clotting system. As shown in Table VII, the minimum amount of salmine which will inactivate 48 inhibitor units of heparin is 0.02 mg. This is in good agreement with the value arrived at in the animal experiments.

*Use of Reaction between Heparin and Protamine in Blood Transfusion*

Compared with the current methods for the preservation and storage of blood to be used in transfusions, which mostly involve defibrination of the blood, the use of heparin seems to offer many advantages. It is an agent by which the chemical and physical properties of blood and of blood cells are altered to a very slight degree only. By the addition of heparin in sufficient quantity blood can be kept fluid for a considerable period. The main drawback of the method would seem to be the danger of certain untoward reactions produced by the heparin (hemorrhages, etc.) when it is injected into the blood stream together with the transfused blood. It was found in several orienting experiments that the protamine reaction described before may be used here with advantage. From dogs weighing between 12 and 15 kilos, 50 cc. of blood were withdrawn, mixed with heparin, and stored in the refrigerator for 96 hours. The blood was then re-injected, followed by the intravenous administration of an appropriate amount of salmine. The results of one experiment follow: Heparin used, 50,000 units. Blood clotting times: before transfusion  $2\frac{1}{2}$  minutes; 10 minutes after transfusion the blood

stayed liquid for over 120 minutes. After the administration of 5.5 mg. of protamine per kilo the clotting time almost immediately fell to 5 minutes. The possibilities of this method are being investigated.

### *General Reactions*

Following the use of heparin the dogs all developed hematomas at the site of injection, regardless of the ease with which the vein was entered. The incidence of hematomas was materially reduced in cases in which protamine was administered. Systemically the smaller doses up to 10,000 units per kilo showed very little effect. Doses of 10,000 units or more were followed in about 5 minutes with retching, vomiting, and involuntary defecation and urination. Occasionally the dogs experienced chills. The symptoms lasted for about 30 minutes after the injection and then subsided completely. No permanent effect was noted.

Following the injection of large doses of protamine there was at times a severe reaction which consisted of weakness, inability to stand, and, rarely, a definite convulsion with opisthotonos. These symptoms subsided within 10 to 15 minutes after the injection. Doses of 3 to 5 mg. per kilo elicited little systemic response. No animals died as a result of the experiments. The administration of the synthetic anticoagulants was followed by almost no reaction.

The hematocrit and hemoglobin readings were almost normal throughout the experiments. The red blood cell counts as a rule did not fall below 4,500,000. The platelet counts were not significantly altered during the course of any of these experiments.

### DISCUSSION

The experiments described in the preceding pages regarding the intravenous administration of heparin, cellulose sulfuric acid, and polyvinyl sulfuric acid have demonstrated that the action of both the natural and synthetic anticoagulants follows a similar course. While the maximum inhibiting effect is reached very rapidly and uniformly in all cases, the duration of the effect is entirely a matter of dosage. The importance of heparin and various synthetic anticoagulants for the prevention of thrombosis will be the subject of further research.

The discovery of the inactivation of anticoagulants by protamine is of manifold interest. For theoretical reasons it should be noted that salmine, which itself is an anticoagulant, has what may be called an activating effect on the clotting of plasma containing heparin. If the explanation which is given for the inhibiting effect of protamines (15), namely that they combine with the thrombin, is right, then one would have to assume that the protamine has a greater affinity for heparin than for the enzyme complex. Compared with the insulin-protamine complex, the combination with heparin seems to be extremely firm. In no case was there observed any activity at all after the administration of protamine, although the testing of blood samples was often continued for many hours. In Experiments 18 and 19 (Table V) and in Experiments 25 and 26 (Table VI) a slight elevation of the blood clotting time could be noted for some time after the administration of salmine. This, however, seems to be within the limit of error of our determinations.

One possible practical application of this reaction has been mentioned before: its use in blood transfusion. The inactivation reaction may also be of value, after heparin has been introduced into clinical use, since it makes it possible to interrupt the heparin action at any desired time. The reaction may furthermore be useful for diagnostic purposes. If there exist types of bleeding or of extended blood clotting time in which heparin is one of the etiological factors, the administration of protamine should bring about a marked change. The reaction will allow recognition of disturbances in which heparin actually plays a rôle. Blood samples from two cases of obstructive jaundice with prolonged coagulation time, which were recently examined, failed to respond to the addition of salmine. The conclusion is justified that in these cases heparin was not the agent which produced the prolonged coagulation time. These studies will be continued.

We acknowledge with thanks the gift of large amounts of purified heparin by Hoffmann-La Roche, Inc., Basel and Nutley, New Jersey. We are indebted to E. R. Squibb and Sons, New Brunswick, New Jersey, and to Eli Lilly and Company, Indianapolis, Indiana, for samples of protamine. In the course of the experiments we have had the assistance of Mrs. Charlotte Breitung and

Mr. Leon Hammer. Miss D. M. Mapes and Mr. Nicholas Buckheim were of valuable assistance in the animal experiments.

## SUMMARY

The mode of action of the anticoagulants heparin, cellulose sulfuric acid, and polyvinyl sulfuric acid has been studied in dogs. It has been found that the anticoagulant effect of heparin and other substances, both *in vitro* and *in vivo*, is destroyed by the protamine salmine. The significance of these findings and possible practical applications are discussed.

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## RADIOACTIVE PHOSPHORUS AS AN INDICATOR OF PHOSPHOLIPID METABOLISM

### I. THE RATE OF FORMATION AND DESTRUCTION OF PHOSPHO- LIPIDS IN THE FASTING RAT\*

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(Received for publication, September 25, 1937)

Attempts to determine the rates of formation and utilization of phospholipids in the body have met with many difficulties. In the early attempts highly unsaturated fats were used, and the changes in the iodine number of the total phospholipid were taken as the index of the rate of turnover. The limitations of this method have already been pointed out (1). It is sufficient to state here that such a method at best can serve only as an index of qualitative changes rather than quantitative changes of total phospholipid in the body, for it has been shown that although the quality of the fat in the diet modifies the nature of the fat incorporated in the phospholipid molecule, no inflexible relation exists between the nature of the fat ingested and the degree of unsaturation of the resulting phospholipids (2). A labeled phospholipid molecule containing elaidic acid has also been employed for measuring phospholipid activity in the animal (1). In this procedure, the elaidic acid content of the phospholipid fraction is measured. While this provides information on the rate at which elaidic acid is incorporated into the phospholipid molecule, it has nevertheless been pointed out by Sinclair (3) that this method does not necessarily give a true index of *total* phospholipid activity, since at present there are no means of knowing what fractions of newly formed phospholipids are present as dielaidyl and monoelaidyl lecithins.

\* Aided by a grant from the Research Board of the University of California.

† Abraham Rosenberg Fellow in Chemistry.

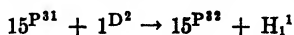


The fact that the radioactive isotope of phosphorus ( $P^{32}$ ) can be readily prepared and has a relatively long life (half life 14.8 days) has made feasible its use as a radioactive indicator in phospholipid metabolism. It was first used for this purpose by Chievitz and Hevesy (4). Since it has not been possible to separate the isotopes of any element in measurable amounts by ordinary chemical procedures (with the exception of hydrogen and deuterium), it appears that  $P^{32}$  is indistinguishable from ordinary phosphorus  $P^{31}$  in its chemical behavior. Although it is known that certain minute separations of isotopes<sup>1</sup> do occur, the magnitude of such separations is far too small to be of practical significance. In a given sample of phosphorus containing  $P^{32}$ , the radioactivity at any instant is a quantitative measure of the labeled phosphorus content. Moreover, once in the tissues of an animal, such a phosphorus sample is marked so long as its radioactivity exists, and this characteristic does not interfere with its chemical behavior until the phosphorus nucleus disintegrates. Similarly, once incorporated in the phospholipid molecule, the latter becomes marked and its history can be followed from formation to destruction. Identification of the phospholipid molecule by radioactive phosphorus possesses distinct advantages over methods in which a foreign fatty acid has been employed, for the former permits the study of phospholipids containing fatty acids normally present in the body. Not only has it been possible to follow phospholipid formation in the presence of ingested fat, but, in addition, this tool has made possible the study of endogenous phospholipid metabolism. The sensitivity of the method is worthy of note, for it is limited only by the amount of  $P^{32}$  that can be produced. The greater the ratio of  $P^{32}:P^{31}$ , the correspondingly greater becomes the sensitivity of the method. In the present investigation, 0.1 mg. of phospholipid has been measured with a fair degree of accuracy. This, however, is by no means the minimum amount detectable, for by the preparation of still stronger samples of radioactive phosphorus it should be possible to measure considerably smaller amounts of phospholipids.

<sup>1</sup> Brewer (5) has recently stated that isotopic separations for potassium occur in certain body tissues.

## EXPERIMENTAL

$P^{32}$  was prepared by bombardment of  $P^{31}$  with deuterons accelerated in the cyclotron of Professor E. O. Lawrence. The following equation represents the nuclear reaction.



After removal from the cyclotron the phosphorus was dissolved in aqua regia, and a solution of the pure disodium phosphate prepared.

Two series of experiments were conducted.

*Series 1*—Twelve male rats, ranging in weight from 190 to 220 gm. and fasted for 30 hours, received 1 cc. of  $Na_2HPO_4$  solutions containing 1.5 mg. of phosphorus (containing 48,300 radioactive units<sup>2</sup>). This was immediately followed by 1 cc. of cod liver oil. All administrations were made by means of a stomach tube. At various intervals up to 75 hours the animals were killed by a blow on the back of the head and immediately ground in their entirety four times to insure uniform sampling. Two portions of approximately 50 gm. each were then placed in alcohol.

*Series 2*—Twenty-four male rats, weighing between 180 and 220 gm., were used. These had been fasted for 40 hours. Twelve of these received 4 mg. of phosphorus each (containing  $1.45 \times 10^5$  radioactive units), in the form of  $Na_2HPO_4$ , along with 1 cc. of cod liver oil. The remainder was fed  $Na_2HPO_4$  only. All these animals were killed, at various intervals, and the following tissues taken for analyses: liver, both kidneys, brain, and gastrointestinal tract. Visible fat was carefully removed from the above tissues and the entire organ or organs cut and immersed in alcohol. The rest of the animal was ground and sampled as in Series 1.

The tissues were extracted with alcohol and ether and concentrated *in vacuo*. The lipids in this concentrate were then quantitatively extracted with petroleum ether. The details of these procedures have been described elsewhere (6). Aliquots of the petroleum ether extract were placed in 50 cc. centrifuge tubes and evaporated to a small volume on a steam bath. The

<sup>2</sup> Arbitrary radioactive units (1 radioactive unit =  $2 \times 10^{-6}$  microcurie).

phospholipid was then precipitated by the addition of acetone and magnesium chloride, the mixture centrifuged, and the acetone decanted.

*Measurement of Labeled Phospholipids*—The samples of precipitated phospholipid were transferred to blotting paper (3 cm.  $\times$  6 cm.) by means of a spatula. The centrifuge tubes were refluxed twice with a few cc. of hot absolute alcohol and the washings transferred quantitatively to the absorbent paper. These were then wrapped with thin cellophane to prevent loss of the adherent phospholipid. Great care was taken to insure uniformity in the mounting of the samples. They were wrapped around a very small Geiger counter (0.1 mm. aluminum wall thickness). The sensitivity of the counter was standardized by means of a thorium source before and after each phospholipid determination. To provide for variations in the amount of phospholipid derived from the various tissues studied, egg lecithin was added to the petroleum ether extract of the smaller samples just before precipitation with acetone. This not only minimized losses of labeled phospholipid in precipitation and transfer but also insured the same percentage absorption of  $\beta$ -rays in the sample itself. In the preparation of the blanks the  $\text{Na}_2\text{HPO}_4$  was mixed with egg lecithin and mounted in the same fashion. To be sure that the samples measured contained  $\text{P}^{32}$  in the form of phospholipid and not inorganic phosphorus that had in some way become occluded in the final precipitate, the following check was performed. To 50 gm. of tissue of a rat (not fed  $\text{P}^{32}$ ), 48,300 radioactive units of  $\text{Na}_2\text{HPO}_4$  solution were added and intimately mixed. Then the total lipids were extracted as above and the acetone-insoluble fraction examined for radioactivity. This showed  $\sim 5$  radioactive units. In an experiment in which  $\text{P}^{32}$  was fed, the acetone solution was examined and found to contain  $\sim 3$  radioactive units, which indicated a quantitative precipitation of the phospholipid.

*Sensitivity of Method*—As noted above, the sensitivity depends upon the ratio of  $\text{P}^{32}:\text{P}^{31}$ . In the sample of phosphorus employed in this investigation there were  $10^9$  to  $10^{10}$  atoms of  $\text{P}^{32}$ . The ratio of  $\text{P}^{32}:\text{P}^{31}$  was less than  $10^{-10}$ . By means of the Szilard-Chalmers<sup>3</sup>

<sup>3</sup> Erbacher and Philipp (8), employing this technique, have succeeded in obtaining  $\text{P}^{32}$  concentrated in a solution containing less than  $4 \times 10^{-7}$  gm. of phosphorus ( $\text{P}^{31} + \text{P}^{32}$ ).

(7) method of preparing concentrated radioactive elements it is possible to make the ratio of  $P^{32}:P^{31}$  any desired magnitude less than  $10^{-3}$  to  $10^{-4}$ . It is unlikely that this ratio can be made greater because of the experimental difficulties. Since the presence of an amount as low as  $10^8$  atoms of  $P^{32}$  can be readily detected by the Geiger counter, it follows that  $10^{-10}$  to  $10^{-11}$  gm. of marked phosphorus (so long as it can be readily isolated for measurement) can be traced within the animal's tissues.

### Results

The results of the first series of experiments in which the phospholipid fraction was followed in the whole animal are shown in Fig. 5 (●). The data obtained in the second series are recorded in Figs. 1 to 6. The ordinates of Figs. 1 to 4 and 6 represent the percentage of fed labeled phosphorus that appeared as phospholipid in the entire organ studied. No distinct differences were noted in the shapes of the curves when the percentage of labeled phosphorus that appeared as phospholipid was plotted per unit weight of a tissue rather than in relation to the entire organ.

It should be stressed here that since only thirty-six animals were used, certain details of Figs. 1 to 3 are not to be regarded as finally settled. Nevertheless, the general shape is unmistakable. Duplicate samples from the extract of each tissue were measured with the Geiger counter, and each point on the curve represents the average of the results obtained from two animals.

*Gastrointestinal Tract. Rats Fed Cod Liver Oil*—The response of the intestine of twelve fasted male rats is shown in Fig. 1. It can be seen that the most rapid synthesis of labeled phospholipid occurs during the first few hours after ingestion of the oil and phosphorus. Thus at an interval of 2 hours after the ingestion of 4 mg. of labeled phosphorus approximately 2 per cent of this element has been incorporated into phospholipid. About 10 hours later the maximum amount (representing 2.5 to 3 per cent of the labeled phosphorus fed) of phospholipid was present. In this figure 100 per cent labeled phosphorus corresponds to 100 mg. of phospholipid.<sup>4</sup>

*Endogenous Phospholipid Synthesis*<sup>5</sup>.—The lower curve in Fig. 1

<sup>4</sup> Calculation based on the percentage of phosphorus in lecithin.

<sup>5</sup> While the term endogenous has been used to refer to phospholipid formation in the absence of the feeding of foodstuffs, it should be noted

shows that the feeding of the oil<sup>6</sup> is not necessary for the initiation of the phospholipid synthesis in rats fasted for this length of time. This observation suggests that phospholipid synthesis is constantly occurring even in the fasted animal. Here the response of the gastrointestinal tract to the ingestion of phosphorus is a rapid deposition of phospholipid, the rate of which is greatest during the first few hours, the maximum being attained about 12 hours after feeding. The difference in the amount of synthesis

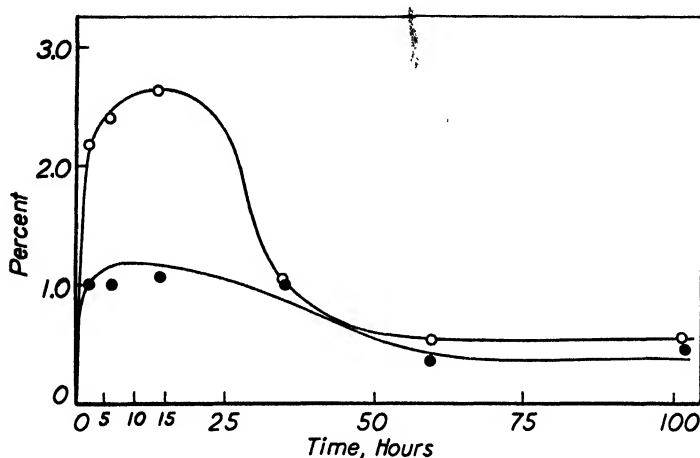


FIG. 1. The labeled phospholipid content of the gastrointestinal tract. The ordinates represent the per cent of fed, labeled phosphorus in the phospholipid of the whole organ. O, obtained from rats fed 1 cc. of cod liver oil along with 4 mg. of labeled phosphorus; ●, obtained from rats that received 4 mg. of labeled phosphorus. 100 per cent on the ordinate scale represents a minimum of 100 mg. of labeled phospholipid. Each point represents the average of four analyses on two rats. This is the case for all the figures.

between the groups of animals fed cod liver oil and those that received only phosphorus is distinct. Thus, while rats that had received the oil along with the phosphorus were able to show a maximum formation of 2 to 3 mg. of phospholipid, the endogenous

that traces of fat that may be still present in the gastrointestinal tract of the fasted animal are not ruled out.

<sup>6</sup> Cod liver oil was the only source of fat used in this study. A comparison of these data with fats containing no vitamin D is in progress.

portion of this (as shown by rats that received only phosphorus) was approximately 1 mg.

Newly synthesized phospholipid, whether formed from endogenous fat or in response to the ingestion of cod liver oil, does not remain long in the tissues of the gastrointestinal tract. The removal (or destruction) of phospholipid from these tissues apparently proceeds at all times. 40 hours after administration of the phosphorus, both curves have reached a plateau, after which the removal of the phospholipid is a slow process. These experiments were discontinued 100 hours after the feeding of oil or phosphorus, or both, and in both series of experiments the amounts of the newly formed phospholipid were not significantly different at the 100 hour mark from what they had been at the 50 hour interval.

*Liver*—The highest concentration of labeled phospholipid occurred in the liver. It is of interest to note that, when cod liver oil was fed, maximum formation occurred in this tissue at an earlier time than in the gastrointestinal tract. The resemblance between the two curves obtained in groups of rats fed either phosphorus alone or phosphorus along with the oil is apparent from Fig. 2. Endogenous phospholipid metabolism therefore proceeds at a rapid rate in this tissue. In the hepatic cell, newly formed or acquired phospholipid is not retained for long, since about 50 hours later the labeled phospholipid content is small.

*Kidney*—Labeled phospholipid is concentrated in the kidneys (Fig. 3) and, although the total amount found in both kidneys is a small fraction of that found either in the entire liver or in the entire intestine, yet, when compared per gm. of tissue, kidney, liver, and intestine contained labeled phospholipid in the same order of magnitude. The same conclusion was reached by Artom *et al.* (9) in a somewhat similar experiment, in which  $P^{32}$  was fed to a single rat. As in the intestinal and liver tissues, here, too, phospholipid deposition takes place in the absence of the feeding of cod liver oil.

*Brain*—The brain showed the slowest as well as smallest response of all tissues examined. The maximum amount of phospholipid was not acquired until about 60 hours after feeding, at which time less than 0.04 mg. of labeled phospholipid was found. It was not possible to carry out complete series of analyses on this tissue, since the radioactivity was too small for accurate measure-

ments. By increasing the  $P^{32}:P^{31}$  ratio, this tissue may be more accurately studied. This will be done in later experiments.

*Carcass*—This term has been used here to include the sum of all tissues not discussed above. Fig. 4 shows that a steady deposition of labeled phospholipid occurs in the carcass. The accumulation of such phospholipid in this tissue residue is slower than that observed in the liver or intestine, but it should be noted that while

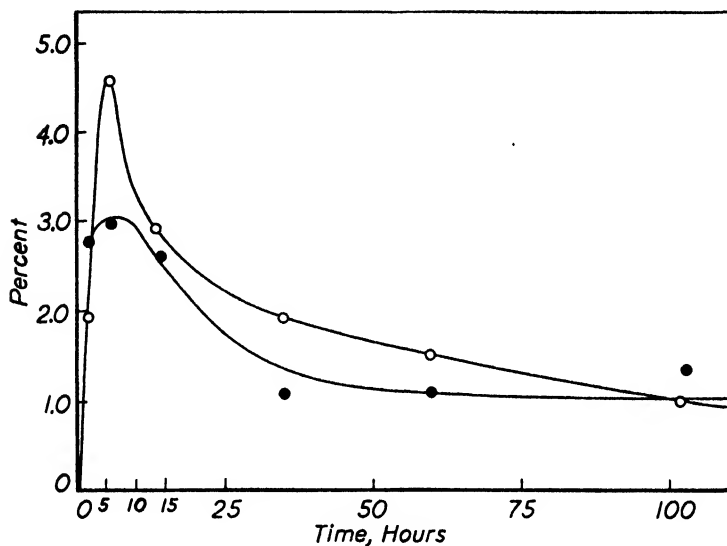


FIG. 2. The labeled phospholipid content of the liver. The ordinates represent the per cent of fed, labeled phosphorus in the phospholipid of the entire liver. The symbols have the same meaning as in Fig. 1. 100 per cent on the ordinate scale represents a minimum of 100 mg. of labeled phospholipid.

a decrease has occurred in the individual tissues studied a steady rise occurs in the carcass. This increase continues even to the time limit of the experiment; namely, 102 hours after feeding. A striking feature of this type of metabolism is the similarity in the two rates followed: one in which phosphorus and cod liver oil were fed, the other in which phosphorus alone was fed. In the carcass tissues endogenous phospholipid metabolism is pronounced, and its rate is apparently not influenced to any great

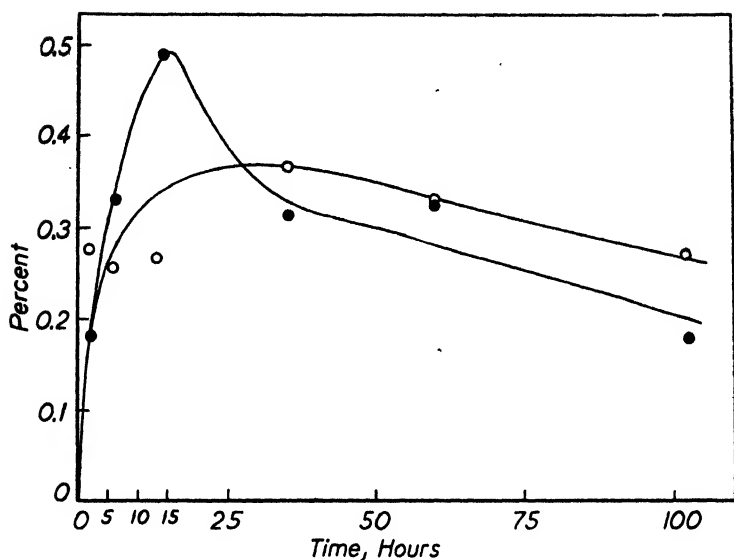


FIG. 3. The labeled phospholipid content of the kidney. The ordinates and symbols have the same meaning as in Fig. 1.

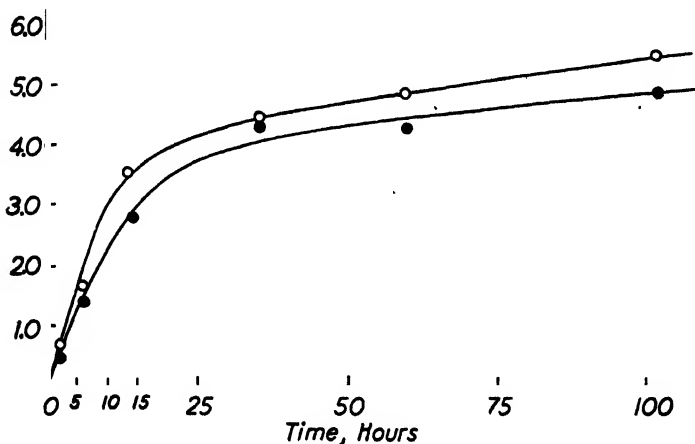


FIG. 4. The labeled phospholipid content of the carcass. The ordinates and symbols have the same meaning as in Fig. 1.



extent by the oil feeding. At the height of the curve this tissue residue contained approximately 5 to 6 mg. of labeled phospholipid.

*Phospholipid Synthesis in Whole Rat*—In the first series of experiments each of the twelve rats received 1.5 mg. of phosphorus along with 1 cc. of cod liver oil, and the entire animal was analyzed for labeled phospholipid. The results are shown by ● in Fig. 5, where the ordinates represent the per cent of the fed phosphorus

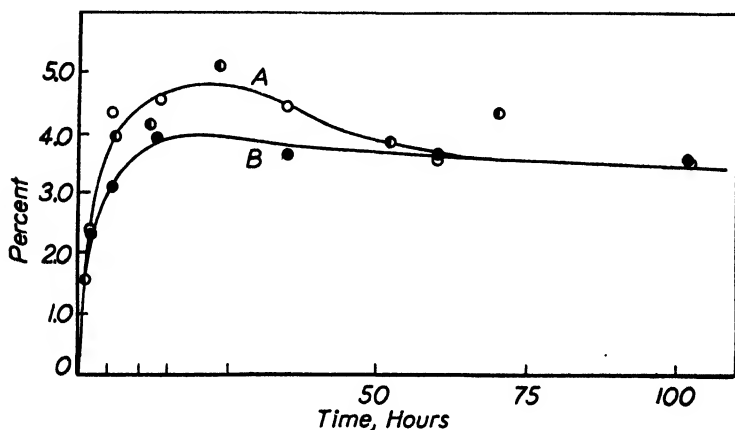


FIG. 5. The labeled phospholipid content of the whole rat. The ordinates represent per cent of fed, labeled phosphorus in the phospholipid per 100 gm. of rat. ●, obtained from Series 1 the rats of which were fed 1 cc. of cod liver oil along with 1.5 mg. of labeled phosphorus; ○, obtained from Series 2, fed 1 cc. of cod liver oil along with 4 mg. of labeled phosphorus; ●, obtained from Series 2, fed 4 mg. of labeled phosphorus only. All points in Series 2 were obtained by summation of the results of analyses of individual tissues.

per 100 gm. of animal that is incorporated in phospholipid. In the second series twelve animals received 4 mg. of phosphorus, and cod liver oil; the phospholipid content is shown by ○ in Fig. 5. Twelve other rats of this second series received 4 mg. of labeled phosphorus; the values for labeled phospholipid are shown by ●. The values represented by ● and ○ were obtained by the summation of all the tissues previously mentioned. Despite the fact that different amounts of phosphorus were fed to the rats in

the two series of experiments, namely 1.5 and 4 mg., the same percentage of fed, labeled phosphorus was found incorporated in phospholipid. Curve A in Fig. 5 was derived from 144 analyses of the tissues of twenty-four rats and would seem to be significant in view of the agreement obtained between the first series shown by  $\bullet$  and the second series shown by  $\circ$ . The former, namely  $\bullet$

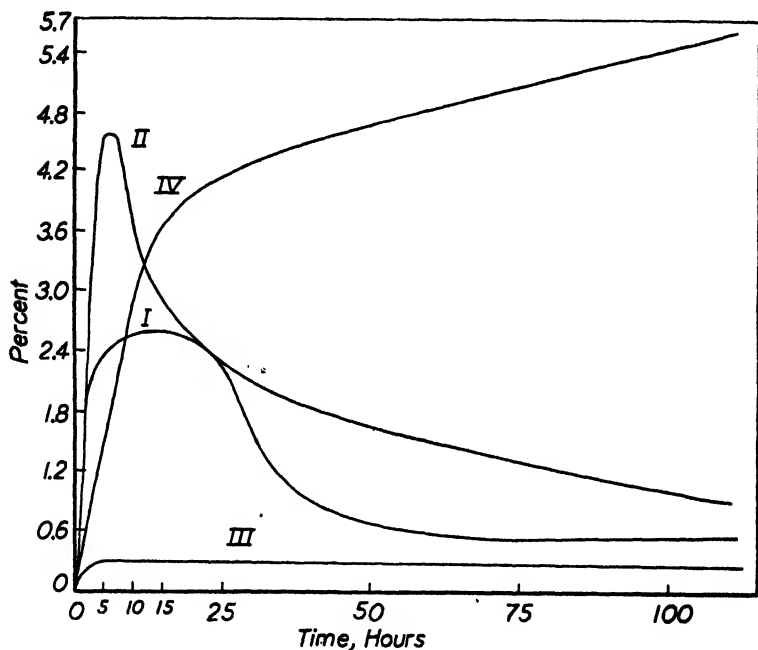


FIG. 6. The relative labeled phospholipid content of the various tissues of the rats fed cod liver oil along with labeled phosphorus. The ordinates represent the per cent of labeled phosphorus found in the entire organs. Curve I represents gastrointestinal tract; Curve II, liver; Curve III, kidney; Curve IV, carcass.

was obtained from analyses of mixed samples of the entire animal, while the latter,  $\circ$ , was derived by summation of values obtained from four separate tissues. There is a high probability, therefore, that Curve A represents an accurate picture of the metabolism of the labeled phospholipid in the presence of ingested cod liver oil. Furthermore, the correlation between the data of the two series

of experiments lends added significance to the results shown for the individual tissues.

Curve B shows the phospholipid synthesis in the whole rats that received phosphorus only. Less phospholipid is formed when cod liver oil is excluded, but the similarity with Curve A is nevertheless apparent. The relative labeled phospholipid content in the various tissues of the fat-fed rats is shown in Fig. 6.

#### DISCUSSION

A study has been made of the response of rat tissues, during early fasting (30 to 40 hours), to the administration of radioactive phosphorus in amounts of 1.5 and 4 mg. Labeled phospholipid appeared in all tissues examined, namely gastrointestinal tract, liver, kidney, and carcass.<sup>7</sup> Similar observations have recently been reported by Artom *et al.* (9). Two phases in phospholipid metabolism were observed: formation (or deposition) and utilization (or removal). A sharp increase in labeled phospholipid content was shown by liver and intestine, while carcass, kidney, and brain showed a slower rise. The rate of disappearance of labeled phospholipid from these tissues decreased in the following order: liver, intestine, and kidney. No decline was observed in the carcass (and possibly the brain) for the duration of the experiment. The question arises whether all tissues in which the labeled phospholipid appeared were capable of synthesis of this lipid constituent or whether synthesis was carried out by one or two of these tissues, the remainder receiving it after formation elsewhere. While the data warrant no certain conclusions, the rates of accumulation, as shown by the curves, suggest that certain tissues, namely liver, intestine, and possibly kidney, participate in the synthesis of phospholipid. The possibility, however, of some of these tissues concentrating rather than synthesizing phospholipid must not be overlooked.

Despite the early rise and fall of labeled phospholipid in the various tissues, Fig. 5 shows that no great changes occur in the

<sup>7</sup> The tissues included in the term "carcass" have been listed above. Blood was not studied in this investigation. Hevesy and Lundsgaard (10), however, have recently shown that labeled phospholipid can be detected in the blood after the administration of radioactive phosphorus.

total amount of labeled phospholipid in the whole animal after maximum synthesis is attained. Thus, 50 hours after feeding, about 80 per cent of the maximum labeled phospholipid synthesized is still present. This does not mean that utilization of phospholipid is not occurring in the body, since it is reasonable to expect that once labeled phospholipid reaches the various tissues (other than liver and intestine, in which it is probably formed), it becomes intimately mixed with unlabeled phospholipid already present. The relatively flat portions of the curves in the later stages of the experiment suggest that a return to the steady state has occurred.

A striking feature of the present study is the synthesis of labeled phospholipid in the absence of oil feeding, a formation which in this paper has been termed endogenous. The various tissues do not show the same capacity for carrying on this type of metabolism. The rate of labeled phospholipid metabolism in the liver is apparently not greatly influenced by the absence of oil feeding, whereas the intestine shows considerably less formation when such feeding is excluded at the time the radioactive phosphorus is administered. While it seems evident that the labeled phospholipid formed in the absence of the feeding of cod liver oil represents a true endogenous synthesis in the liver, no such conclusion can be drawn in the case of the intestine, for here the small effect observed may have been due to traces of fat still present in the intestinal tract of the fasted animal.

We are indebted to Professor E. O. Lawrence and members of the Radiation Laboratory for the preparation of the radioactive samples of phosphorus that made this study possible. Our thanks are also due to Mr. B. A. Fries for technical assistance. This investigation was aided by a grant to the Radiation Laboratory from the Josiah Macy, Jr., Foundation.

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## STUDIES ON THE VITAMIN B COMPLEX\*

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(Received for publication, September 28, 1937)

New developments in the vitamin B complex have recently appeared in such rapid succession that any summary of its status would be incomplete almost before it had been printed. Without further introduction, this paper briefly records the results obtained in a series of experiments on the unknown factors in the hope that the observations will be useful to others studying the same subject as theirs have been to us.

### EXPERIMENTAL

The growth of rats and their skin lesions were the methods of assay employed. The basal diet was designed to contain no water-soluble vitamins that are present in the common sources of protein and carbohydrate such as casein and starch.

The adequacy of purified casein as a source of protein was tested because earlier work (2) had shown that the nutritive value of commercial casein could be lowered by heating or by extraction with 95 per cent alcohol. Since this might possibly be due to an alteration of the protein itself as well as to the removal of accessory factors, the biological value (Mitchell) of casein after several types of treatment was determined. The biological value of crude (commercial) casein, 73, was not lowered by (a) heating for 2 hours at 120°, 76, (b) extraction for 4 days with hot 95 per cent alcohol, 71, (c) both extraction and heating, 74. A similar value, 72, was found for casein prepared from milk by isoelectric precipitation and purified by long extractions with acidulated water (2 weeks), alcohol (1 week), and ether (1 week). These values are the average figures obtained from eight to ten animals in alternating rota-

\* Presented in part at the meeting of the American Society of Biological Chemists at Memphis, April, 1937 (1).

tion on each kind of casein, and the complete data are available. Alteration of the protein apparently is not responsible for the nutritional inadequacy of various purified caseins.

Crude casein contains some of all of the factors necessary for the rat with the exception of thiamine chloride (vitamin B<sub>1</sub>) (see Table I, A). Riboflavin is present in suboptimal amounts. After a time three of the five animals developed crusted lips and noses, indicating insufficient quantities of other factors.

TABLE I  
*Growth of Rats on Caseins with Various Supplements*

Daily supplements*	No. of rats	Initial weight	Final weight	Weight gain	Time	
A. Diet containing crude casein						
	♂	♀	gm.	gm.	gm.	days
Flavin + B <sub>1</sub> .....	2	3	49	88	39	40
B <sub>1</sub> .....			88	111	23	90
None.....			111	94	-17	5
B. Diet containing purified casein						
B <sub>1</sub> , flavin, physin concentrate.....	4	4	45	70	25	35
“ “ K-P B <sub>1</sub> .....	3	4	48	55	7	30
“ “ K-P B <sub>4</sub> .....	3	4	47	67	20	35
“ “ heat-treated yeast.....	4	4	46	132	86	40
“ heat-treated yeast.....	2	3	92	125	33	15
“ “ “ extract.....	3	3	67	99	32	15
“ only.....	3	3	99	99	0	20

\* The supplements were as follows: Flavin, 13 mg. (the flavin was Booher's flavin, prepared from whey powder as described in the text); B<sub>1</sub>, crystalline thiamine chloride, 7.5 micrograms; physin equivalent to 2.0 gm. of fresh liver; K-P B<sub>1</sub> concentrate equivalent to 10 to 20 gm. of fresh yeast; K-P B<sub>4</sub> concentrate equivalent to 2.5 to 5.0 gm. of fresh yeast.

Purified isoelectric casein was first used in the diets but later commercial casein which had been extracted for 4 days with boiling alcohol in a percolator was substituted. Responses of animals on either casein seemed the same. Hydrogenated cottonseed oil<sup>1</sup> was used as the source of fat, since it also supplied vitamin E which has some influence on later growth. The possible presence of a water-soluble vitamin in this fat (3) must be considered in a final analysis of our results.

<sup>1</sup> Crisco.

The basal diets contained the following ingredients: casein, 18 per cent; cystine, 0.4; sucrose, 55.1; salts (Hawk and Oser (4)), 4.5; fat, 20; cod liver oil, 2. Cystine was added to avoid a possible deficiency in the casein. When thiamine chloride was supplied it was given in daily doses of 7.5 micrograms of Merck's crystalline preparation either by mouth or together with other daily supplements separate from the basal diet. In the earliest experiments riboflavin was supplied as a concentrate prepared from whey powder by the method of Booher (5). The concentration was carried only through the chloroform-ethyl alcohol extraction.

Three concentrates were first tested to see if any one of them alone would permit growth when fed along with thiamine chloride and riboflavin (Table I, B).

*Physin*—In view of the possible identity of physin (6) and the Coward factor (7) a liver concentrate was prepared according to Mapson's method and tested as a supplement to thiamine chloride and riboflavin. A fair growth rate was obtained.

*Peters' Eluate*—This vitamin B<sub>1</sub> concentrate was shown by György (8) to contain vitamin B<sub>6</sub>. As prepared from fresh starch-free yeast (Fleischmann) by the method of Kinnersley, O'Brien, Peters, and Reader (9), and here designated K-P B<sub>1</sub>, it produced very poor growth when supplemented with thiamine chloride and riboflavin.

*Vitamin B<sub>4</sub>*—Reader's vitamin B<sub>4</sub> (10), here called K-P B<sub>4</sub>, was obtained from yeast during the preparation of the K-P B<sub>1</sub> concentrate (9), and permitted rats to grow at a fair rate when supplemented with thiamine chloride and riboflavin. This growth was comparable with that obtained with physin but much better than that with K-P B<sub>1</sub> even though a smaller equivalent of yeast was given.

From these results it appears that some other factor (or factors) besides thiamine chloride, riboflavin, and vitamin B<sub>6</sub> is necessary to complete the diet. This is present in the K-P B<sub>4</sub> concentrate and in physin but not to any great extent in the K-P B<sub>1</sub> concentrate.

*Heat-Treated Yeast*—On the assumption that the unknown factor is possibly heat-stable, dried yeast<sup>2</sup> was autoclaved for 6 hours

<sup>2</sup> We are indebted to Northwestern Yeast Company for liberal supplies of dry yeast.



at 15 pounds pressure and then heated dry at 120° for 36 hours. When given in daily doses of 0.5 gm. to young rats receiving thiamine chloride and riboflavin concentrate, the growth rate was over 2 gm. per day (Table I and Fig. 1, Group IV at *A*). Apparently the unknown factor and vitamin B<sub>6</sub> are stable to moist and dry heating. The riboflavin is likewise, since animals receiving only thiamine chloride in addition to the heat-treated yeast grew as well as those receiving riboflavin also (Group II at *E* and Table I.) A concentrate prepared by extracting the heat-treated yeast four times with boiling 80 per cent alcohol was equally as effective (Group II at *B* and Table I) as the heat-treated yeast itself.

*Fractionation of Heat-Treated Yeast Extract*—Since the feeding trials and the fluorescence of the extract showed that the heat-treated yeast contained large amounts of riboflavin, an attempt was made to remove it by adsorption. Bringing a concentrated extract to pH 1.0 with hydrochloric acid preliminary to adsorption produced a heavy red-brown precipitate. Treating the clear filtrate at pH 1.0 with three portions of fullers' earth left a slightly yellow solution designated non-flavin extract. The washed fullers' earths were combined and eluted with a pyridine-methanol-water mixture. This fluorescent eluate was designated flavin extract. In all experiments with the heat-treated yeast and fractions thereof, except the acid precipitate, daily doses equivalent to 0.5 gm. of yeast were fed.

Animals fed the acid precipitate with thiamine chloride failed to grow (Fig. 1, Group I at *M* and *N*). The yeast residue remaining after the alcoholic extraction, when fed with thiamine chloride, permitted maintenance of weight (Group IV at *I*), thus indicating

FIG. 1. Growth rate of rats on the vitamin B complex. The roman numbers represent groups; the figures in parentheses, initial and final weights. The supplements are represented by letters as follows: *A*, vitamin B<sub>1</sub> + flavin (13 mg.) + treated yeast; *B*, B<sub>1</sub> + treated yeast extract; *C*, B<sub>1</sub>; *D*, B<sub>1</sub> + flavin (13 mg.) + non-flavin extract; *E*, B<sub>1</sub> + treated yeast; *F*, B<sub>1</sub> + flavin (39 mg.) + non-flavin extract; *G*, B<sub>1</sub> + flavin extract + non-flavin extract; *H*, B<sub>1</sub> + non-flavin extract; *I*, B<sub>1</sub> + yeast residue; *J*, B<sub>1</sub> + flavin extract; *K*, B<sub>1</sub> + flavin extract + yeast residue; *L*, B<sub>1</sub> + non-flavin extract + yeast residue; *M*, B<sub>1</sub> + precipitate from extract (0.5 gm. of yeast); *N*, B<sub>1</sub> + precipitate from extract (1.0 gm. of yeast); *O*, B<sub>1</sub> + flavin (13 mg.) + yeast residue; *P*, B<sub>1</sub> + flavin (26 mg.) + yeast residue.

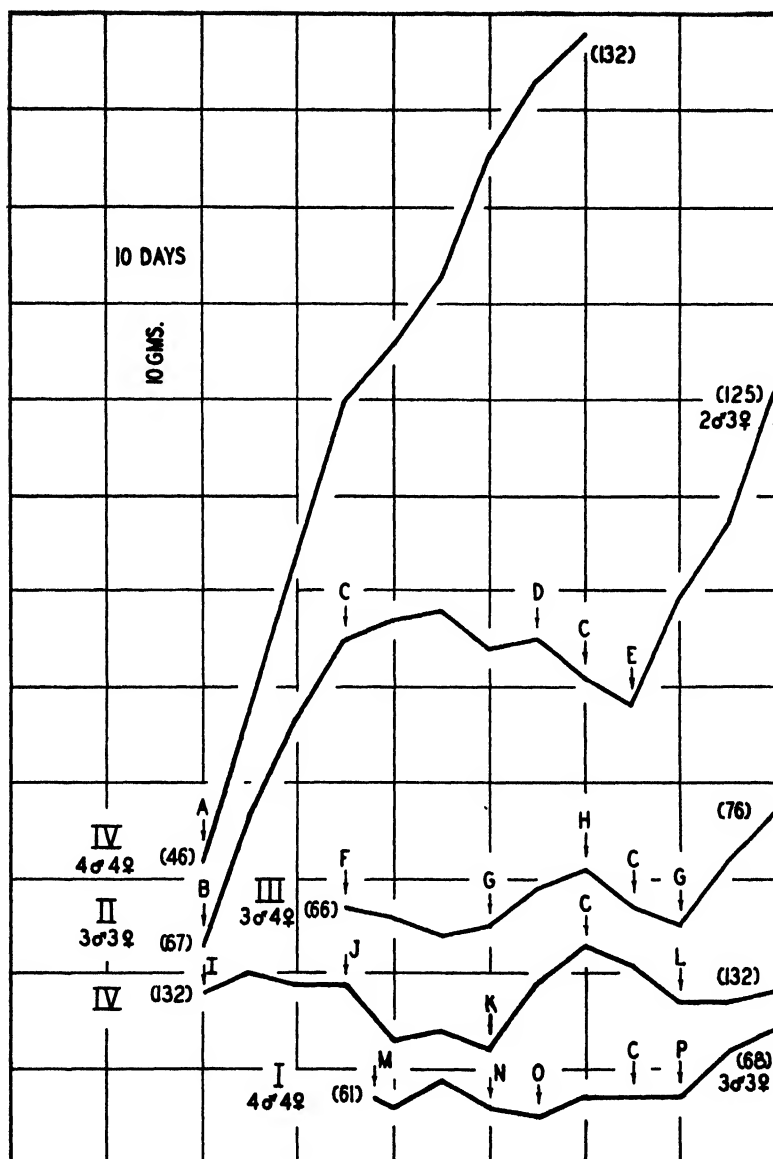


FIG. 1

that some activity still remained there. Apparently more of the adsorbable than of the non-adsorbable factors were extracted (Group I at *O* and *P*, Group IV at *K* and *L*).

The flavin extract when given with thiamine chloride produced loss in weight (Group IV at *J*). So did the non-flavin extract (Group III at *H*). However, when the two were fed together (Group III at *G*), growth was restored, but at a slower rate than with the unfractionated extract. This loss in total activity was probably through manipulation; the combined flavin and non-flavin portions had the qualitative properties of the whole extract.

Although animals grew when the flavin extract was fed together with the non-flavin extract (Group III at *G*), the Booher flavin preparation with the non-flavin extract resulted in a loss of weight even when 39 mg. of the Booher concentrate were supplied daily (Group II at *D* and Group III at *F*). In the adsorbable material some factor other than riboflavin was therefore present, and this factor was not present in the concentrate prepared from whey powder.

*Effect of Light on Heated Yeast Extract*—Hogan and Richardson (11) reported a differentiation between the antidenuding and the antidermatitic factors by means of irradiation. The former (riboflavin) was destroyed by visible or ultraviolet light, while the latter was destroyed only by the ultraviolet. In our hands this method failed to provide an identification or segregation of the factors in heat-treated yeast extract, perhaps because of inadequate exposure to visible light (two 200 watt lamps at 6 inches for 24 hours). Exposure to a mercury vapor light at 6 inches for 15 hours destroyed about half the growth-promoting activity. The factors for growth present in yeast extract are apparently not as sensitive to light as the reports in the literature would lead one to suspect. Further experience indicated that reasonable care in preventing too much exposure to light gave consistently reliable results.

*Whole Yeast Fractions as Supplements to Small Amounts of Heated Yeast*<sup>3</sup>—If the factors in yeast are not equally sensitive to heat, supplementing the heat-treated yeast with concentrates

<sup>3</sup> One of us (H. W. S.) conducted these experiments during the summer of 1936 at Colorado College in Colorado Springs, Colorado. We are indebted to Professor R. J. Gilmore and to Colorado College for granting facilities.

of the various factors might yield semiquantitative information as to the extent to which each of them is inactivated. Elvehjem and Koehn (12), working with chicks, fractionated liver extract by adsorption into two components, riboflavin and a factor remaining in the filtrate which they called vitamin B<sub>2</sub> (designated "filtrate factor" by Lepkovsky and Jukes (13)). Later Elvehjem, Koehn, and Oleson (14) reported a principle necessary for the rat (precipitate factor) in an alcohol-ether precipitate obtained in the course of this separation. After an 80 per cent alcoholic extract of dried yeast was treated twice with fullers' earth at pH 1.0, the filtrate was fractionated into alcohol-ether insoluble (Y-4) and soluble (Y-9) portions by the method of these authors (12, 14). The

TABLE II

*Growth of Rats with and without Supplements Added to 0.25 Gm. of Heat-Treated Yeast Daily*

Daily supplements* (55th to 85th days)	Initial weight	Weight at 55 days	Weight at 85 days	Weight change (30 days)
	gm.	gm.	gm.	gm.
B <sub>1</sub> (thiamine chloride) 7.5 micrograms . . .	40	99	121	22
" + 22.5 micrograms B <sub>1</sub> . . . . .	39	98	127	29
" + Y-4 . . . . .	43	101	130	29
" + Y-9 . . . . .	39	96	119	23
" + Y-3 . . . . .	42	100	145	45

\* Yeast fractions fed were equivalent to 1 gm. of dry yeast.

soluble material was purified by successive treatments with acetone, ether, and amyl alcohol and should have contained the filtrate factor. The washed fullers' earths were eluted with pyridine-methanol-water mixture to give fraction Y-3.

Young rats were fed 0.25 gm. of heat-treated yeast and 7.5 micrograms of crystalline thiamine chloride daily for 55 days. Five groups were then compounded to give an even distribution as to sex and gain in weight during this period in which the animals had been supplied with suboptimal amounts of the growth factors. Each group contained six or seven animals.

During the succeeding 30 days, in which various further supplements were given (Table II), the differences in growth rates were not striking but the animals receiving fraction Y-3 showed the

greatest gains. From this it appears that the essential material most easily destroyed by heat is adsorbed on fullers' earth at pH 1.0. The alcohol-ether-soluble (Y-9) and insoluble (Y-4) materials are little affected by the heat treatment. The response to the adsorbate was not due to additional thiamine chloride, since this by itself did not measurably improve the growth rate.

There is an alternative explanation for these results. If the filtrate factor in yeast is thermolabile, as has been claimed by some (15), then it is not necessary for growth in the rat, for it is certainly not adsorbed by fullers' earth. If the precipitate factor is thermolabile, then the very slight activity of concentrate Y-4 may be due to the adsorption of that factor on fullers' earth at pH 1.0.

*Experiments with Factor 1 and Factor 2*—To substantiate one or the other of the above views concentrates of Factors 1 and 2 of Lepkovsky, Jukes, and Krause (16) were prepared from air-dried yeast essentially by the method of those authors for making preparations from liver and rice bran extracts. 2 kilos of yeast were extracted three times with 6 liter portions of boiling 80 per cent alcohol. The combined extracts were concentrated *in vacuo* to 550 cc. and the solids and the fats removed by centrifugation and ether extraction. When diluted to 800 cc., the pH was 4.8. The solution was then treated twice with 100 gm. portions of fullers' earth and three times with 80 gm. portions. The filtrate was concentrated *in vacuo* to 200 cc. for feeding as Factor 2. From the fullers' earth used in the second adsorption a concentrate of Factor 1 was prepared.

Results obtained by supplementing the basal diet with various combinations are summarized in Table III. The animals on thiamine chloride, crystalline riboflavin, Factor 1, and Factor 2 (Lot 4) grew fairly well. Substituting heat-treated yeast for either Factor 1 or Factor 2 (Lots 7 and 8) produced slightly better growth, but when heat-treated yeast replaced the riboflavin (Lot 6) the growth rate was much slower. Best growth was shown by the animals fed heat-treated yeast in addition to thiamine chloride, riboflavin, Factor 1, and Factor 2 (Lot 5), suggesting that heat-treated yeast may contain yet another factor beyond those four. However, optimal amounts of Factors 1 and 2 may not have been fed in this series of experiments. The differences in

growth rates indicate that Factors 1 and 2 are present in the heat-treated yeast in rather large amounts; the riboflavin had been partially inactivated.

The various skin lesions provided further information as to the nature of these supplements. Rats which received no riboflavin (Lot 1) merely maintained their weights and all developed rough

TABLE III

*Growth of Rats on Various Combinations of Thiamine Chloride, Riboflavin, Factor 1, Factor 2, and Heat-Treated Yeast*

Lot No.	Supplements*	Average weight	No. of rats	Average weight	No. of rats	Weight gain	Average weight	No. of rats	Weight change
		Initial		40 days			80 days		
		gm.		gm.		gm.	gm.		gm.
1	B <sub>1</sub> , Factor 1, Factor 2	39	6	46	6	7	36	1	-3
2	" riboflavin " 2	44	6	84	6	40	90	6	46
3	" " " 1	44	7	58	7	14	78	3	34
4	" " " 1, Factor 2	67	8	114	8	47	128	8	61
							50 days		
5	B <sub>1</sub> , heat-treated yeast, riboflavin, Factor 1, Factor 2	65	8	128	8	63	143	8	78
6	B <sub>1</sub> , heat-treated yeast, Factor 1, Factor 2	66	8	95	8	29	99	8	33
7	B <sub>1</sub> , heat-treated yeast, riboflavin, Factor 2	66	9	126	9	60	136	9	70
8	B <sub>1</sub> , heat-treated yeast, riboflavin, Factor 1	66	9	123	9	57	135	9	69

\* Daily doses were as follows: B<sub>1</sub> (thiamine chloride) 7.5 micrograms; crystalline riboflavin, 10 micrograms; heat-treated yeast, 0.5 gm.; Factors 1 and 2 equivalent to 1 gm. of yeast per day.

and somewhat thinned coats. Some riboflavin was present in the concentrates of Factors 1 and 2 because weight was maintained and there was no denuding. The basal diet, when fed to young rats with crystalline thiamine chloride only, produces almost complete loss of hair in 2 weeks.

Rats which received no Factor 1 concentrate (Lot 2) grew fairly

well, but in 50 to 70 days four of the six animals developed vitamin B<sub>6</sub> lesions around the mouth, nose, and eyes. Those which received no Factor 2 concentrate (Lot 3) grew at a much slower rate and each of the surviving three rats developed "spectacled eyes." Although the growth of these two groups and the delayed production of symptoms show that neither concentrate contains one factor free from the other, there is an approximate separation of the two by fullers' earth adsorption at pH 4.8. The slower growth rate of Lot 2 and the faster rate of Lot 3 after the 40th day may be due to the use of new preparations of Factors 1 and 2 during that period. An English fullers' earth was used in the later experiments in place of one of unknown origin.

Except for absence of lesions on the ears, these observations suggest that Factor 1 is the vitamin B<sub>6</sub> of György as Lepkovsky and his coworkers believe. Since our experiments are concerned only with rats, they do not show conclusively whether Factor 2 is the filtrate factor as required by the chick or not. If the precipitate factor is also a necessary component of the vitamin B complex for the rat, as Elvehjem *et al.* believe, it must have been supplied with either Factor 1 or Factor 2.

#### DISCUSSION

These experiments have shown that at least four water-soluble vitamins are required by the rat. Two of these, thiamine chloride and riboflavin, were supplied as crystalline preparations. The other two, Factor 1 and Factor 2, were used in the form of fairly pure concentrates prepared from yeast. Factor 1 is apparently vitamin B<sub>6</sub> and Factor 2 is either the precipitate factor or the filtrate factor.

No evidence has been presented here to show that vitamin B<sub>4</sub> (Reader) is not required by the rat. If the dietary fat<sup>1</sup> contained this vitamin, as Kline, Bird, Elvehjem, and Hart (3) believe, then it was supplied to all of our animals. The growth of rats fed crystalline thiamine chloride and riboflavin (Booher) together with either the K-P B<sub>4</sub> concentrate or the physin concentrate may have been due to the vitamin B<sub>4</sub> in these latter two preparations. The experiments with Factors 1 and 2 more logically suggest that these preparations contained, in addition to vitamin B<sub>6</sub>, the active constituent of Factor 2.

An active filtrate containing Factor 2 could be prepared from an extract of air-dried yeast adsorbed with fullers' earth at pH 4.8. When adsorption was carried out at pH 1.0, however, a purified concentrate of the filtrate factor was inactive; the alcohol-ether-insoluble material obtained during purification (which should contain the precipitate factor) was only slightly active. One might therefore conclude that the filtrate factor is not required by the rat and that most of the precipitate factor had been adsorbed at pH 1.0. Our method for demonstrating the inactivity of a purified concentrate of the filtrate factor is valid, because Keenan *et al.* (15) and Lepkovsky and Jukes (13) have shown that this factor *as required by the chick* is inactivated in yeast and dietary foodstuffs by dry heat.

A fullers' earth adsorbate prepared from yeast extract at pH 1.0 would therefore contain riboflavin, vitamin B<sub>6</sub>, and the active constituent of Factor 2. Prepared at pH 4.8, it would contain only riboflavin and vitamin B<sub>6</sub>. By analogy the same principle probably holds for adsorptions on charcoal; the greater activity of the K-P B<sub>4</sub> concentrate over that of the K-P B<sub>1</sub> concentrate in our first experiments appears to be due to its greater content of both vitamin B<sub>6</sub> and the constituent of Factor 2.

A summary, by no means complete, of reported adsorptions for the various factors of the vitamin B complex is given in Table IV. Obviously, success in the satisfactory adsorption of any one factor depends upon the physical and chemical state of the factor in the solution, the reaction of the solution, and the adsorbent (see Halliday and Evans (22) and Birch and György (21)).

Although riboflavin and vitamin B<sub>6</sub> are apparently adsorbed at any acid pH, no evidence is at hand for adsorption of either the filtrate factor or the precipitate factor on fullers' earth. Recently Edgar and Macrae (24) have reported that a rat factor remains in the filtrate after an autoclaved yeast extract is treated with fullers' earth at pH 1.4, 8, or 10. This confirms our observation that heat-treated yeast extract, after adsorption at pH 1.0, yields an active filtrate. Being unable, however, to prepare active concentrates of either the filtrate factor or the precipitate factor after adsorption (at pH 1.0) of an unheated yeast extract, we believe that the active constituent of Factor 2 essential to the rat shows a differential adsorption according to the reaction of the solution and



the state in which the factor exists in the heated and unheated sources. Differences in fullers' earths may also be concerned here.

Contamination of fullers' earth eluates with Factor 2, in the hands of Lepkovsky, Jukes, and Krause (16), may have been due to adsorption as they suggest; had they adsorbed from a more

TABLE IV  
*Adsorption of Vitamin B Complex*

Factor	Adsorption on fullers' earth	Adsorption on charcoal	Authority
Thiamine chloride	pH 4.5 (yeast) " 4.5 (rice polish)	pH 7.0 (yeast)  Slightly, pH 1 (yeast)	Guha (17) Williams, Waterman, and Keresztesy (18) Kinnersley, O'Brien, Peters, and Reader (9) " "
Riboflavin	pH 1.0 (milk, etc.)  pH 1-8 (liver)		Kuhn, György, and Wagner-Jauregg (19) Lepkovsky, Popper, and Evans (20)
Vitamin B <sub>6</sub> (Factor 1)	" 2.5 and 5.0 (wheat germ) Not at pH 9.0 (wheat germ) pH 4.0 (liver dialysate) pH ? (liver)  " 5.2-5.4* (rice bran)	pH 7.0 (yeast) Not at pH 6.0 (herring extract)	Birch and György (21) " "  Halliday and Evans (22) Lepkovsky and Jukes (23) Lepkovsky, Jukes, and Krause (16) György (8) Birch and György (21)
Filtrate factor (chick)	Not in acid (liver) Not at pH 5.6-5.8* (liver)	No, pH ? (liver)	Elvehjem and Koehn (12) Lepkovsky, Jukes, and Krause (16) Lepkovsky and Jukes (13)

TABLE IV—*Concluded*

Factor	Adsorption on fullers' earth	Adsorption on charcoal	Authority
Filtrate factor (rat)	Not at pH 5.6- 5.8* (liver)		Lepkovsky, Jukes, and Krause (16)
	Not at pH 1.4, 8, 10 (heated yeast)		Edgar and Macrae (24)
		pH 1.2, 2.5 (heated yeast)	" "
		Slightly, pH 7, 8.2 (heated yeast)	" "
	Not at pH 1.0 (heated yeast)		Present report
	pH 1.0 (yeast)		" "
Precipitate factor (rat)	Not at pH 4.8 (yeast)		" "
		pH 1.0 (yeast)	" "
		Slightly, pH 7.0 (yeast)	" "
Vitamin B <sub>4</sub>	No, pH ? (liver)		Frost and Elvehjem (25)
		pH ? (liver)	Elvehjem, Koehn, and Oleson (14)
		" 1.0 (yeast)	Kinnersley, O'Brien, Peters, and Reader (9)
		Slightly, pH 7.0 (yeast)	" "

\* Personal communication from T. H. Jukes.

acid solution, all of Factor 2 (the rat essential) might have been removed. Our experiments certainly show that separation of the two factors is incomplete by their method.

The filtrate factor as assayed with chicks is not adsorbed by charcoal (13). The precipitate factor, however, is adsorbed (14), and so is the factor discussed by Edgar and Macrae. This fact, together with our demonstration of the inactivity of a concentrate of the filtrate factor and variances in reports on heat stability, suggests that Factor 2 probably contains two essentials, one required by the rat, the other by the chick. It must be added, however, that the properties given by Edgar and Macrae for the rat factor conform quite well with those for the chick factor as given by Lepkovsky and Jukes (13).

Although we obtained very poor growth with the Peters' eluate (K-P B<sub>1</sub>), György (8) was able to produce fairly good growth with thiamine chloride, riboflavin, and Peters' eluate when a vitamin B-free basal diet was used. Knowing, as we do now, that thiamine chloride, riboflavin, and vitamin B<sub>6</sub> are not the only factors necessary for the rat, the Peters' eluate used by György must have contained the active constituent of Factor 2 along with vitamin B<sub>6</sub>; our own preparation contained small amounts. This factor could be present in the eluate because Edgar and Macrae have shown that it is not precipitated by lead acetate and that it is adsorbed slightly on charcoal at pH 7.0.

György (8) has also found that the Bourquin-Sherman diet (containing wheat extract) supplemented with riboflavin will not permit rats to grow. However, if either the Peters' eluate or a yeast extract from which the riboflavin has been removed by fullers' earth was also supplied, growth did result. These experiments indicate that some factor is adsorbed on charcoal but not on fullers' earth. The limiting factor here seems not to be vitamin B<sub>6</sub>, which is probably present in the wheat extract, but rather the constituent of Factor 2.

According to a recent report by Booher (26), her whey powder concentrate (vitamin H) contains the combined growth and anti-dermatitis factors other than thiamine chloride and riboflavin. When we fed this preparation with thiamine chloride and K-P B<sub>1</sub> or with thiamine chloride and the non-flavin extract of heat-treated yeast, there was little or no growth. No suitable explanation of this discrepancy can be offered at present.

#### SUMMARY

1. By careful adsorption of an 80 per cent alcohol extract of yeast it has been demonstrated that besides thiamine chloride and riboflavin at least two additional factors are necessary for growth and well being in the rat.

2. One of these, Factor 1, is the "vitamin B<sub>6</sub>" of György and is necessary for growth and prevention of dermatitis. It is adsorbed on fullers' earth at pH 4.8 and 1.0 from a yeast extract.

3. The second of these, Factor 2, is probably the "precipitate factor" of Elvehjem, Koehn, and Oleson, and is essential for growth. It is adsorbed from an unheated yeast extract by fullers'

earth at pH 1.0 but not completely at pH 4.8. As present in an extract of heat-treated yeast it appears not to be adsorbed by fullers' earth. With charcoal it is adsorbed at pH 1.0 and slightly at pH 7.0.

4. "Vitamin B<sub>2</sub>" (filtrate factor) of Elvehjem and Koehn is not necessary for growth in the rat. A concentrate of Factor 2 probably contains it.

5. Both Factor 1 (vitamin B<sub>6</sub>) and Factor 2 (precipitate factor), as present in dried yeast, are stable to autoclaving for 6 hours at 15 pounds pressure followed by dry heating for 36 hours at 120°.

6. Riboflavin, as present in dried yeast, is less stable to the same heat treatment.

7. A summary and discussion of the adsorption characteristics of the components of the vitamin B complex are given.

8. By various methods of purification commercial casein can be made more suitable for use in basal diets for studies on the vitamin B complex without altering its nutritive value as a protein. The biological value was not lowered by (a) extraction for 4 days with hot 95 per cent alcohol, (b) heating for 2 hours at 120°, (c) extraction and heating. The same biological value was found for casein prepared from milk by isoelectric precipitation and purified by long extractions with acidulated water, alcohol, and ether.

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## CONVERSION OF URONIC ACIDS INTO CORRESPONDING HEXOSES

### II. CATALYTIC REDUCTION OF THE METHYL ESTER OF 2,3,4-TRIMETHYL $\alpha$ -METHYL-*D*-GALACTURONIDE

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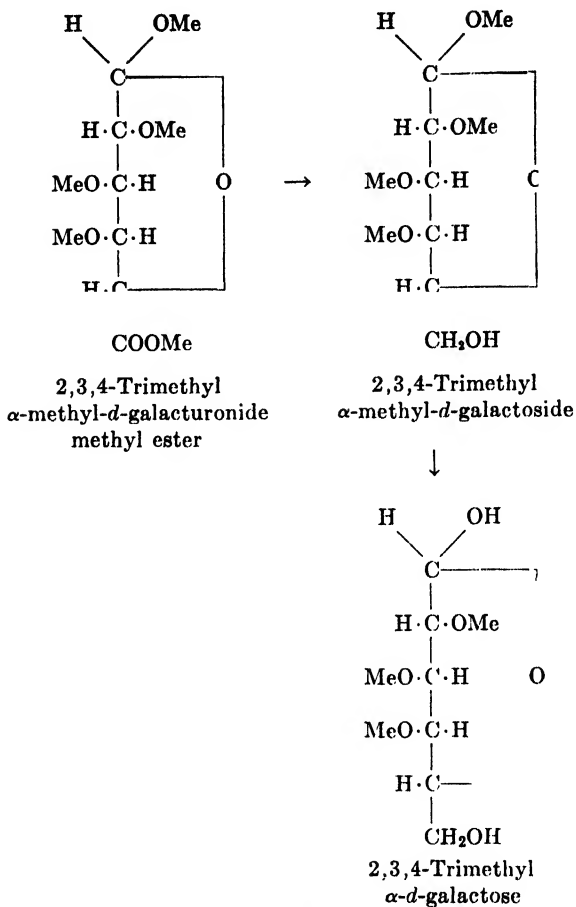
In the first communication of this series<sup>1</sup> is described the conversion of the methyl ester of 2,3,4-trimethyl  $\alpha$ -methyl-*D*-galacturonide to 2,3,4-trimethyl  $\alpha$ -methyl-*D*-galactoside by a series of transformations, at the terminal carbon atom, involving the following steps: ester  $\rightarrow$  amide  $\rightarrow$  nitrile  $\rightarrow$  amine  $\rightarrow$  carbinol.

Since the final yield of the 6-hydroxy derivative was necessarily not theoretical, owing to the number of stages passed through, a simpler and more direct process was sought. We have now found that the ester may be converted directly to the 6-hydroxy derivative, in good yield, by treatment with hydrogen in the presence of copper chromite catalyst.

The method provides a "tool" which may prove of service in the elucidation of the structure of more complex derivatives of uronic acids, including the pectins and aldobionic acids. As was discussed in the first communication, the use of the methods of reduction of uronic acids to the corresponding sugars was developed for the purpose of application to the analysis of structure of polysaccharides either composed entirely of uronic acid residues, or containing a uronic acid residue as one of their components.

<sup>1</sup> Levene, P. A., and Kreider, L. C., *J. Biol. Chem.*, **121**, 155 (1937).

## 200 Uronic Acids to Corresponding Hexoses



### EXPERIMENTAL

*2,3,4-Trimethyl  $\alpha$ -methyl-*D*-galacturonide methyl ester*<sup>2</sup> had  $n_D^{25} = 1.4558$  (in the superfused state) and displayed the following specific rotations.

$$[\alpha]_D^{27} = \frac{+3.82^\circ \times 100}{2 \times 1.150} = +166.1^\circ \text{ (in water)}$$

$$[\alpha]_D^{27} = \frac{+3.43^\circ \times 100}{2 \times 1.100} = +155.9^\circ \text{ (in absolute methanol)}$$

<sup>2</sup> Levene, P. A., and Kreider, L. C., *J. Biol. Chem.*, **120**, 597 (1937).

$$[\alpha]_D^{25} = \frac{+3.18^\circ \times 100}{2 \times 1.114} = +142.7^\circ \text{ (in chloroform)}$$

$$[\alpha]_D^{25} = \frac{+3.23^\circ \times 100}{2 \times 1.082} = +149.3^\circ \text{ (in acetone)}$$

$$[\alpha]_D^{25} = \frac{+3.53^\circ \times 100}{2 \times 1.060} = +166.5^\circ \text{ (in } n\text{HCl; unchanged after 4 days at room temperature)}$$

*Hydrogenation of 2,3,4-Trimethyl  $\alpha$ -Methyl-d-Galacturonide Methyl Ester*—4.5 gm. of 2,3,4-trimethyl  $\alpha$ -methyl-d-galacturonide methyl ester were dissolved in 100 cc. of absolute methanol, 4 gm. of copper chromite catalyst<sup>3</sup> were added, and the mixture was shaken in an atmosphere of hydrogen, at a pressure of 4300 pounds per sq. inch, during 5 hours at 175°.

The mixture was now cooled and the major portion of the catalyst removed by centrifuging. The solution was evaporated to dryness, the product dissolved in dry ether, and the remaining trace of catalyst filtered off. The filtrate was evaporated to dryness under diminished pressure and the resulting colorless syrup was distilled under a high vacuum.

It distilled completely at about 140° (bath temperature) at 0.3 mm. as a colorless, homogeneous, viscous syrup having  $n_D^{25} = 1.4626$  and the following composition.

5.308 mg. substance:	9.926 mg. CO <sub>2</sub> and 4.090 mg. H <sub>2</sub> O
3.299 " "	33.66 cc. 0.01 N sodium thiosulfate
C <sub>10</sub> H <sub>20</sub> O <sub>6</sub> .	Calculated. C 50.81, H 8.6, OCH <sub>3</sub> 52.55
	Found. " 50.98, " 8.6, " 52.75

It displayed the following specific rotations.

$$[\alpha]_D^{25} = \frac{+4.73^\circ \times 100}{2 \times 1.192} = +198.4^\circ \text{ (in water)}$$

$$[\alpha]_D^{25} = \frac{+3.82^\circ \times 100}{2 \times 1.188} = +160.8^\circ \text{ (in absolute methanol)}$$

The syrup was stirred and allowed to stand overnight in the refrigerator; it crystallized in long needles and rapidly set to a mass of crystals, having a melting point of about 30°.

*Hydrolysis of 2,3,4-Trimethyl  $\alpha$ -Methyl-d-Galactoside*—1.0 gm. of trimethyl methylgalactoside was dissolved in 30 cc. of N hydro-

<sup>3</sup> Adkins, H., and Connor, R., *J. Am. Chem. Soc.*, **53**, 1091 (1931).



## 202 Uronic Acids to Corresponding Hexoses

chloric acid and the solution heated at 98–100° under a reflux during 2 hours.

The product was then isolated as described for the preparation of 2,3,4-trimethyl *d*-ribose from trimethyl methylriboside,<sup>4</sup> giving a practically quantitative yield of a colorless syrup which crystallized from solution in a little ethyl acetate when pentane was added to faint opalescence.

The crude sugar (m.p. 70°) was recrystallized from dry ether. After one recrystallization it had a melting point of 78–79°; a second such purification gave a pure product having a melting point of 82–83° (uncorrected),  $n_D^{25} = 1.4810$  (for the superfused substance), and the following composition.

4.219 mg. substance:	7.540 mg. CO <sub>2</sub> and 3.100 mg. H <sub>2</sub> O
3.921 “ “ :	31.83 cc. 0.01 N sodium thiosulfate
C <sub>9</sub> H <sub>18</sub> O <sub>6</sub> .	Calculated. C 48.62, H 8.2, OCH <sub>3</sub> 41.90
	Found. “ 48.73, “ 8.2, “ 41.97

It had the following specific rotation in water

$$[\alpha]_D^{25} = \frac{+3.42^\circ \times 100}{2 \times 1.096} = +156.0^\circ \text{ (3 min. after dissolution)}$$

changing to +151.9° (5 min.), +146.0° (9 min.), +144.2° (10 min.), +143.2° (11 min.), +139.6° (15 min.), +136.9° (20 min.), +125.9° (40 min.), +122.7° (50 min.), +120.9° (60 min.), and +119.1° (90 min.), constant thereafter.

<sup>4</sup> Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **93**, 623 (1931).

## CONVERSION OF URONIC ACIDS INTO CORRESPONDING HEXOSES

### III. CATALYTIC REDUCTION AND DEACETYLATION OF THE METHYL ESTER OF 2,3,4-TRIACETYL $\alpha$ -METHYL-*D*-GALACTURONIDE

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(Received for publication, October 20, 1937)

The practical significance of the reduction of uronic acid derivatives to the corresponding sugar derivatives has been discussed in two previous communications.<sup>1,2</sup> The conversion of the methyl ester of 2,3,4-trimethyl  $\alpha$ -methyl-*D*-galacturonide has been accomplished by methods of classical organic chemistry as well as by the catalytic method. However, the methylation of uronic acids is, in itself, a laborious and tedious process. A convenient method of demethylation has not as yet been developed. Hence it was desirable to develop a method giving direct formation of a monoside from the corresponding uronide.

The reduction of the methyl ester of  $\alpha$ -methyl-*D*-galacturonide catalytically (under conditions employed for the methylated substance) was therefore attempted. However, the reduction apparently progressed too far, so that not only the—COOCH<sub>3</sub> group but also some of the hydroxyl groups were reduced. The reduction product gave a negative test with naphthoresorcinol and reduced Fehling's solution only after hydrolysis. On the other hand, the ratio of carbon to hydrogen in it was much too high for a methylhexoside. It is probable that conditions could be found to arrest the reduction at the desired point. However, instead of searching for such conditions, it was thought expedient

<sup>1</sup> Levene, P. A., and Kreider, L. C., *J. Biol. Chem.*, **121**, 155 (1937).

<sup>2</sup> Levene, P. A., Tipson, R. S., and Kreider, L. C., *J. Biol. Chem.*, **122**, 199 (1937-38).

to test the behavior of the methyl ester of 2,3,4-triacetyl  $\alpha$ -methyl-*d*-galacturonide with the expectation that the acetyl groups likewise would be reduced, so that the resulting substance would be the deacetylated product ( $\alpha$ -methyl-*d*-galactoside).

The expectation was actually realized. The substance was identified by its specific rotation, by its melting point, and by the mixed melting point with an authentic sample of  $\alpha$ -methyl-*d*-galactoside. Furthermore, on hydrolysis of the substance, a product was formed from which galactose phenylosazone was obtained.

It may be mentioned here that the senior author, in cooperation with Dr. R. S. Tipson, has made preliminary experiments on the catalytic reduction of the methyl ester of hexaacetyl  $\alpha$ -methylaldobionide. The crystalline aldobionic acid was obtained from gum arabic by the procedure of Heidelberger. On reduction a product was obtained which no longer contained the carboxyl group, giving a negative naphthoresorcinol test. It reduced Fehling's solution only after hydrolysis. It was, however, analytically slightly impure. The preparation of a purer substance is now in progress.

We may take this occasion also to mention that the glycosides of uronic acids, on boiling with a solution of barium hydroxide, do not form the characteristic orange-yellow precipitate which is generally obtained on boiling uronic acids in the same solvent.<sup>3</sup> This behavior is mentioned, since it offers a means of differentiation between aldobionic acids of the two types: one in which the monose is linked with carbon atom (1) of the uronic acid and the other in which the uronic acid is linked to carbon atom (1) of the monose.

#### EXPERIMENTAL

5 gm. of copper chromite<sup>4</sup> catalyst were added to a solution of 5 gm. of the methyl ester of triacetyl  $\alpha$ -methyl-*d*-galacturonide<sup>5</sup> in 100 cc. of absolute methanol. This mixture was placed in a

<sup>3</sup> On inquiry we have learned from Dr. C. Niemann that identical behavior of glycosides of uronic acids was observed by the group of workers associated with Dr. K. P. Link.

<sup>4</sup> Adkins, H., and Connor, R., *J. Am. Chem. Soc.*, **53**, 1091 (1931).

<sup>5</sup> Morell, S., and Link, K. P., *J. Biol. Chem.*, **108**, 763 (1935).

high pressure reduction apparatus and a hydrogen pressure of 3000 pounds per sq. inch was applied. The temperature was now slowly increased to 175°, the pressure subsequently rising to 4300 pounds per sq. inch. After the reaction had proceeded during 5 hours at this temperature with shaking, the whole apparatus was allowed to cool for 18 hours.

The reaction mixture was removed, some charcoal added, and the catalyst separated by filtration. The filtrate was concentrated to dryness and from an alcoholic solution of the resulting sirup a crystalline material was obtained on addition of a small amount of ether. The crystals were filtered off (yield 1.7 gm.) and then recrystallized from absolute ethanol. A constant melting point of 111–112° was obtained after five such recrystallizations. It then had the following specific rotation.

$$[\alpha]_D^{25} = \frac{+8.80^\circ \times 100}{2 \times 2.30} = +191.4^\circ \text{ (water)}$$

A mixed melting point with an authentic specimen of  $\alpha$ -methyl-*d*-galactoside, having a specific rotation of  $[\alpha]_D^{25} = +192.5^\circ$  (water), showed no depression. The composition of the substance agreed with that of a methylhexoside.

5.324 mg. substance: 8.495 mg. CO<sub>2</sub> and 3.525 mg. H<sub>2</sub>O  
 3.211 " " : 10.47 cc. 0.01 N sodium thiosulfate  
 C<sub>7</sub>H<sub>14</sub>O<sub>6</sub>. Calculated. C 43.30, H 7.3, OCH<sub>3</sub> 15.98  
 Found. " 43.51, " 7.4, " 16.86

The alcohol-ether mother liquor from the first recrystallization was concentrated to a sirup which was hydrolyzed with boiling 3 per cent hydrochloric acid during 2.5 hours. From this solution an osazone was prepared which had a melting point of 202° and a composition agreeing with that for a hexosazone.

3.710 mg. substance: 0.513 cc. N<sub>2</sub> (755 mm. at 28°)  
 C<sub>18</sub>H<sub>22</sub>O<sub>4</sub>N<sub>4</sub>. Calculated, N 15.64; found, N 15.57



# **SOME OBSERVATIONS ON THE KAPELLER-ADLER METHOD FOR THE DETERMINATION OF HISTIDINE. THE HISTIDINE CONTENT OF YEAST\***

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(Received for publication, October 11, 1937)

The Kapeller-Adler method (1) for the determination of histidine has, in the last few years, been widely used. Csonka (2) applied it to yeast hydrolysates and concluded that acid hydrolysis of whole yeast resulted in the destruction of histidine. Histidine concentrates from such hydrolysates did not give the test, while similar concentrates from yeast protein hydrolysates readily responded.

In our work on the diamino acids of the mold *Aspergillus sydowi* it was noted that concentrates prepared in a similar manner from the autolyzed mycelium gave only slight, or negative, Kapeller-Adler tests. However, considerable histidine was isolated from such concentrates (3). These results indicated the presence of some substance interfering with the test in the mold concentrates, and suggested that the same condition might obtain in those from yeast hydrolysates. That this was the case was shown by the fact that, when histidine was added to concentrates prepared from yeast by Csonka's method, the Kapeller-Adler test was still negative. Final evidence for the fact that interfering substances and not destruction of histidine explained failure of the test was that considerable histidine could be isolated from such concentrates.

For application to materials such as hydrolysates of whole organisms we have modified the Kapeller-Adler procedure in

\* This work was supported in part by a grant from the Special Research Fund of the Graduate School.

several details. The use of an excess of bromine, as recommended by Conrad and Berg (4), eliminated reduction in the color caused by flavin, and the excess of the former was conveniently removed by aeration before the addition of ammonia. Contrary to the views of Kapeller-Adler, excess bromine in itself does not inhibit the reaction, but the excess must be removed before any alkali is added. Furthermore, since the solutions examined absorbed considerable bromine, a saturated solution of the latter was used in place of the usual 1 per cent reagent in order to keep the volume of the test solution at a convenient level. Finally, since interfering substances precipitable by  $\text{AgNO}_3$  or  $\text{HgSO}_4$  are not precipitated by phosphotungstic acid, this reagent, along with  $\text{AgNO}_3$  or  $\text{HgSO}_4$ , has been used to purify the solutions for analysis. Phosphotungstic acid alone did not entirely separate histidine from interfering substances. Several amino acids that inhibit the reaction are precipitated by silver and mercury salts.

#### EXPERIMENTAL

*Modified Procedure*—The solution to be examined is treated with  $\text{AgNO}_3$  and  $\text{Ba(OH)}_2$  at pH 7.4, or with  $\text{HgSO}_4$  in 5 per cent  $\text{H}_2\text{SO}_4$  if reducing sugars are present. The histidine is freed from the precipitate with  $\text{H}_2\text{S}$ , and the filtrate from the sulfide is concentrated under reduced pressure and treated with phosphotungstic acid. The phosphotungstate precipitate is decomposed with  $\text{Ba(OH)}_2$ , and excess barium is removed from the filtrate as  $\text{BaSO}_4$ . To 1 cc. of the resulting acid solution, which should contain approximately 1 mg. of histidine, a saturated solution of bromine in 33 per cent acetic acid is added dropwise until a deep yellow color persists. After the mixture has stood for 10 minutes, excess bromine is removed by bubbling air through it until the yellow color disappears. The determination is then completed as described by Kapeller-Adler.

*Interfering Substances*—Kapeller-Adler recognized that excess bromine inhibited the formation of color, and attributed this effect to further reaction of bromine with histidine. But the inhibiting action is probably due to conversion of the histidine by  $\text{NH}_4\text{OBr}$  (formed when the ammonia is added) to the corresponding aldehyde or cyanide containing 1 less carbon atom (5). In

support of this theory, we have found that if a reagent prepared by neutralizing the bromine solution with the ammoniacal solution was added to histidine before the test was applied, no color was produced.

A number of amino acids and nitrogen bases were tested for their effect on the test as modified above.  $\alpha$ -Aminobutyric acid, leucine, isoleucine, norleucine, serine, phenylalanine, lysine, arginine, cystine, aspartic acid, glutamic acid, guanine, or adenine was without effect. Tyrosine, tryptophane, or uracil gave brown colors which prevented comparison with the standard. Glycine or methionine markedly inhibited the reaction, and proline, hydroxyproline, or alanine considerably reduced the color. When 2 mg. of glycine were added to a solution of 1 mg. of histidine and the test was performed, only 50 per cent of the color produced by 1 mg. of histidine was formed. A similar amount of alanine reduced the color by 25 per cent, while a similar amount of  $\alpha$ -aminobutyric acid had no appreciable effect. It was of interest to note that the two substituted alanines, serine and cystine, had no effect. Proline or hydroxyproline (2 mg. per mg. of histidine) reduced the color about 25 per cent. A rather brown color was produced in all cases by the interfering amino acids mentioned. In  $\text{HgSO}_4$  and  $\text{AgNO}_3$  precipitates of solutions prepared from whole organisms, uracil or tyrosine, or both, may be present, but these interfering substances can be separated from histidine with phosphotungstic acid.

*Isolation of Histidine from Hydrolyzed Yeast*—500 gm. of dried bakers' yeast were hydrolyzed for 21 hours at  $120^\circ$  with 2500 cc. of 8.5 N  $\text{H}_2\text{SO}_4$ , and then most of the  $\text{H}_2\text{SO}_4$  was removed as  $\text{BaSO}_4$ . The filtrate and washings were concentrated under reduced pressure to 1 liter, treated with excess  $\text{HgSO}_4$  in 5 per cent  $\text{H}_2\text{SO}_4$ , and allowed to stand in the cold for 48 hours. The solution resulting from the decomposition of the precipitate with  $\text{H}_2\text{S}$  was concentrated under reduced pressure to 150 cc., the histidine was precipitated with  $\text{AgNO}_3$  and  $\text{Ba(OH)}_2$  at pH 7.4 in the usual manner, and the precipitate of histidine silver was decomposed in  $\text{H}_2\text{SO}_4$  solution with  $\text{H}_2\text{S}$ . After removal of the sulfide, the solution was concentrated under reduced pressure to 100 cc. and treated with 10 gm. of flavianic acid. After 2 weeks the crystals



which had slowly formed were filtered off and recrystallized from dilute flavianic acid. 5.69 gm. of yellow crystals melting at 250–252° with decomposition were obtained.

$C_{25}H_{21}O_{13}N_7S_2 \cdot \frac{1}{2}H_2O$ . Calculated, S 8.08; found, S 8.10

The hydrochloride was prepared from the diflavianate by butyl alcohol extraction of the flavianic acid, and was recrystallized from dilute alcoholic HCl. M.p. 245°.

$C_6H_9O_2N_3 \cdot 2HCl$ . Calculated,  $NH_2-N$  6.14; found,  $NH_2-N$  6.15

The diflavianate separated out very slowly, and it is possible that much more histidine was present than was actually isolated.

From the histidine diflavianate filtrate after removal of reagents 890 mg. of tyrosine were obtained.

*Histidine Content of Yeast*—25 gm. of the same batch of yeast that was used above were hydrolyzed as outlined above, and the concentrated hydrolysate, after removal of most of the  $H_2SO_4$ , was treated with phosphotungstic acid and placed in the refrigerator for 24 hours. The solution resulting from the decomposition of the phosphotungstate precipitate was concentrated under reduced pressure to about 25 cc. and treated with  $AgNO_3$  and  $Ba(OH)_2$  at pH 7.4. When the precipitate so produced was decomposed in dilute  $H_2SO_4$  with  $H_2S$ , the filtrate from the  $Ag_2S$  was found by analysis with the modified Kapeller-Adler method to contain 1.03 gm. of histidine per 100 gm.

*Recovery of Added Histidine from Yeast*—25 gm. of yeast and 80 mg. of histidine monohydrochloride (65 mg. of histidine) were hydrolyzed and the hydrolysate was fractionated with  $HgSO_4$  and  $AgNO_3$  as described above. The histidine solution from the decomposition of histidine silver precipitate was treated with phosphotungstic acid, and the precipitate produced was decomposed in the usual manner. 321 mg. of histidine were found by analysis of the resulting solution, or a recovery of 97 per cent of the histidine added.

#### SUMMARY

1. The use of excess bromine in the Kapeller-Adler determination of histidine as described by Conrad and Berg, and the re-

moval of the excess by aeration before the ammoniacal reagent is added have been found advisable.

2. A number of amino acids and naturally occurring nitrogenous substances have been tested for their effect on the color produced in the Kapeller-Adler reaction. Tryptophane, tyrosine, uracil, proline, hydroxyproline, methionine, glycine, or alanine interferes, owing either to reduction in the amount of color produced or to the formation of brown color that obscures the test.

3. It is recommended that histidine be precipitated with phosphotungstic acid as well as with silver or mercury before applying the Kapeller-Adler reaction.

4. Histidine is not destroyed during acid hydrolysis of yeast.

5. The yeast examined contained 1.03 per cent histidine.

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## THE CHEMISTRY OF MOLD TISSUE

### XIV. ISOLATION OF CYCLIC CHOLINE SULFATE FROM ASPERGILLUS SYDOWI\*

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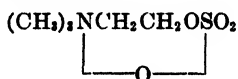
During the investigation of the nitrogenous compounds elaborated from glucose and inorganic salts by *Aspergillus sydowi* (1-3) a white crystalline substance was isolated which contained sulfur and nitrogen. This was obtained from the mercury acetate-sodium carbonate filtrate of an autolysate of the mold pad (2) by precipitation with saturated phosphotungstic acid and treatment with alcohol; the sulfur-containing compound appeared to be the only organic substance in such filtrates which was precipitable by phosphotungstic acid and was practically insoluble in alcohol. Considerable loss during isolation resulted from the use of the former reagent, for the substance was precipitated by phosphotungstic acid only from rather concentrated solutions. In fact, when the usual concentrations of reagents and solution were employed it was not precipitated, but could be isolated from the filtrate by laborious fractional crystallization. The substance was also isolated from the mercury acetate-sodium carbonate filtrate of an acetone extract of defatted mycelium (1), and hence was probably present free in the mycelial pad.

The crystalline material was only sparingly soluble in alcohol and various organic solvents, but was readily soluble in water, yielding a neutral solution. It was optically inactive, and possessed no melting point. Qualitative tests showed the presence of sulfur and nitrogen, and analyses led to the empirical formula  $C_5H_{13}O_4NS$ . No ash was present. The compound did not react

\* This work was supported in part by a grant from the Special Research Fund of the Graduate School.

with acetyl chloride, phenylhydrazine, or diazomethane, and gave no nitrogen with nitrous acid in the Van Slyke determination. Although it was obtained by crystallization from alcohol containing HCl, no chlorine was present in the molecule. When it was boiled with 40 per cent NaOH, the odor of trimethylamine was readily detected, and examination of the chloroplatinate of the volatile base confirmed its presence. The original compound gave no precipitate with HCl and BaCl<sub>2</sub>, but after the alkaline cleavage these reagents immediately precipitated BaSO<sub>4</sub>. No ether-soluble matter was found in the distillation residue after this alkali treatment.

At this point, a search of the literature showed that Schmidt and Wagner (4) had synthesized a compound of the formula C<sub>8</sub>H<sub>18</sub>O<sub>4</sub>NS from choline chloride and concentrated H<sub>2</sub>SO<sub>4</sub>, and had assigned to it the structure



They found that boiling HCl liberated choline and H<sub>2</sub>SO<sub>4</sub>. Since the description of the properties of the above compound coincided with those observed for the isolated material, acid hydrolysis was attempted on the latter, and from the hydrolysate H<sub>2</sub>SO<sub>4</sub> and choline were readily obtained. Cyclic choline sulfate was then synthesized according to the directions of Schmidt and Wagner and found to possess the same crystalline structure, solubility characteristics, and behavior with phosphotungstic acid as the isolated material. Unfortunately, since the compounds possessed neither optical activity nor melting point, more direct comparison was not possible, but it was felt that the results obtained by alkaline degradation and by acid hydrolysis left no doubt as to the identity of the isolated substance. So far as we are aware, cyclic choline sulfate has never before been isolated from natural substances.

The amount of recrystallized substance obtained was 0.26 per cent of the dry mycelium. Undoubtedly there was much more present, for the isolation procedure involved considerable loss. When saturated phosphotungstic acid was used to precipitate it, losses resulted owing to the solubility of the phosphotungstate,

and when its isolation was attempted from a phosphotungstic acid filtrate made under the ordinary conditions of precipitation, the presence of large amounts of NaCl and mannitol (removed by fractional crystallization) reduced the yield.

#### EXPERIMENTAL

The starting point in this investigation was the mercury acetate-sodium carbonate filtrate prepared as previously described (2)



FIG. 1. Cyclic choline sulfate isolated from *Aspergillus sydowi*;  $\times 100$

from 525 gm. of dry mycelium, and a similar filtrate prepared from the acetone extract of 1 kilo of defatted mycelium (1). The former filtrate had a volume of 7 liters and contained 3.9 gm. of nitrogen. To it was added concentrated HCl until it was acid to Congo red and then mercury was removed with  $\text{H}_2\text{S}$ . The filtrate and washings from the  $\text{HgS}$  were concentrated under reduced pressure to about 500 cc. and 1 liter of hot alcohol was added. Insoluble matter, mainly NaCl, was filtered off and washed with alcohol, and alcohol was removed from the filtrate

under reduced pressure. To the concentrated aqueous solution which remained, a saturated solution of phosphotungstic acid was added until no more precipitate formed and the mixture was placed in the refrigerator overnight. Glistening white crystals with a metallic sheen separated out above the usual rather amorphous phosphotungstate precipitate. The solid matter was centrifuged out and decomposed in the usual manner with barium hydroxide. The filtrate from barium phosphotungstate was made acid with  $\text{H}_2\text{SO}_4$ , and sulfate was then removed with  $\text{BaCl}_2$ . The resulting filtrate was concentrated to dryness under reduced pressure, the residue was digested with about 300 cc. of alcohol, and the insoluble, inorganic residue discarded. The filtrate was concentrated to dryness under reduced pressure, dissolved in the minimum amount of hot alcohol, and allowed to stand in the refrigerator for 24 hours. The beautiful white crystals which separated out were filtered off, washed with alcohol, and recrystallized from alcohol; 1.36 gm. were obtained. The compound crystallized in either plates or rods as shown in Fig. 1. The plates seemed to predominate if crystallization proceeded slowly.

$\text{C}_{12}\text{H}_{13}\text{O}_4\text{NS}$ .	Calculated.	C 32.9, H 7.1, N 7.65, S 17.5
	Found.	" 32.8, " 6.9, " 7.30, " 17.3

*Isolation from Acetone Extract*—Mercury and  $\text{NaCl}$  were removed from the mercury acetate-sodium carbonate filtrate of the acetone extract of defatted mycelium (1) as described above for the corresponding filtrate of the autolysate. Choline was then precipitated with iodine dissolved in strong  $\text{NaI}$  solution, and the resulting filtrate was freed of iodine with "molecular" copper and  $\text{CuCl}_2$  (5), and then of copper with  $\text{H}_2\text{S}$ . The solution was then treated with 10 per cent phosphotungstic acid solution, the precipitate was removed, and the filtrate was freed of reagents and concentrated to dryness under reduced pressure. Three recrystallizations of the residue from alcohol yielded pure cyclic choline sulfate.

$\text{C}_{12}\text{H}_{13}\text{O}_4\text{NS}$ .	Calculated, N 7.65; found, N 7.43
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*Phosphotungstate*—Both the synthetic and isolated cyclic choline sulfates were precipitated from 10 per cent aqueous solu-

tion by saturated phosphotungstic acid in  $N H_2SO_4$  as lustrous crystals. When viewed under the microscope, these crystals were seen to be very thin, transparent plates. Tests on the synthetic material showed that the concentration of the compound must be between 6 and 7 per cent before any precipitate was produced by adding saturated phosphotungstic acid.

*Alkaline Degradation*—15 mg. were refluxed with 6 cc. of 40 per cent NaOH, and the volatile base formed was aspirated into dilute HCl. When the latter was evaporated, a white crystalline residue remained which was dissolved in alcohol. On the addition of alcoholic  $H_2PtCl_6$  an orange precipitate formed which was found to melt at  $218-220^\circ$  with decomposition. Trimethylamine chloroplatinate in the same bath melted at  $220^\circ$  with decomposition. If the temperature of the bath was raised rapidly the melting point of the chloroplatinates was considerably higher; this may explain the divergent melting points recorded in the literature (6). When the alkaline residue from the degradation experiment was acidified with HCl and treated with  $BaCl_2$ ,  $BaSO_4$  immediately precipitated. The filtrate from the  $BaSO_4$  was evaporated and the residue extracted with ether, but the ether extracts contained no solids.

*Acid Hydrolysis*—20 mg. were heated with 2 cc. of 6  $N$  HCl at  $100^\circ$  for 11 hours and then a slight excess of  $BaCl_2$  was added, and the  $BaSO_4$  centrifuged off. The clear centrifugate was concentrated to dryness under reduced pressure, and the residue was extracted with alcohol. When  $H_2PtCl_6$  was added to the extracts, 33 mg. of orange crystals separated which melted at  $234-235^\circ$  with decomposition. Choline chloroplatinate melts at  $234^\circ$  with decomposition.

$C_{10}H_{22}O_2N_2PtCl_6$ . Calculated, Pt 31.7; found, Pt 31.8

#### SUMMARY

Cyclic choline sulfate has been isolated from the mycelium of *Aspergillus sydowi* and identified by analysis and by the examination of suitable degradation products. 0.26 per cent of the dry mycelium was obtained as this compound, but since there were losses due to the solubility of the phosphotungstate, probably considerably more was present. The substance was obtained



both from an autolysate and from an acetone extract of defatted mycelium. Apparently this sulfuric acid derivative has not been recognized before in natural materials.

We wish to express our thanks to William Saschek who performed the carbon, hydrogen, and sulfur analyses, and to Mark Stahman who prepared the photomicrograph. We are indebted to Dr. E. J. Crane for his advice regarding the naming of the compound.

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# THE RÔLE OF CYSTINE, METHIONINE, AND HOMOCYSTINE IN THE NUTRITION OF THE RAT\*

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It has been known for many years that sulfur-containing amino acids are essential constituents of a properly balanced diet for animals. One of the earliest contributions to the problem of the sulfur requirement in nutrition, made in 1915 by Osborne and Mendel (1), demonstrated that *l*-cystine supplements were capable of stimulating the growth rate of albino rats ingesting a casein-containing diet of low sulfur content. This observation, which has been repeatedly confirmed, led to the general conclusion that cystine was an essential amino acid. More recently, Jackson and Block (2) established the fact that *dl*-methionine is as efficient as *l*-cystine in producing growth in animals stunted by a sulfur-low, casein-containing diet. This discovery was indicative of the existence of a reciprocal function of the two amino acids in nutrition. However, in view of the structural differences between the two compounds, it seemed possible that cystine and methionine might not be completely interchangeable in the diet. The extent to which one of the sulfur-containing amino acids might replace the other has hitherto not been determined, probably because of the difficulties involved in the preparation of suitable experimental diets for definitely establishing the nutritional relationships between cystine and methionine.

In the present investigation, a diet has been devised which contains a considerable amount of cystine but which is apparently

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deficient in methionine. It has therefore been possible to study the effect of added cystine, cysteine, methionine, and homocystine on the growth rate of albino rats ingesting a cystine-high, methionine-low diet.

#### EXPERIMENTAL

*Basal Diets*—Arachin, the principal globulin of the peanut (*Arachis hypogaea*), was chosen as the protein component of the methionine-deficient diet. A search of the literature revealed that of all the proteins thus far analyzed for methionine, arachin contains the lowest amount of this amino acid (0.5 per cent) (3). Moreover, it has been reported by Sure (4) that arachin is unable to support normal growth in animals when used as the chief source of dietary nitrogen, notwithstanding the fact that analyses of arachin indicate the presence of relatively high concentrations of most known essential amino acids.

The arachin employed in the present study was prepared from raw peanuts by the method of Johns and Jones (5) and incorporated into a basal diet having the following composition: arachin 15, starch 55, Crisco 21, Osborne and Mendel salt mixture (6) 4, and cod liver oil 5 per cent. Male rats at weaning were confined singly in suitable cages and given this arachin diet *ad libitum*. In addition to the basal diet, each animal received a daily supplement of 100 mg. of ryzamin B (Burroughs Wellcome and Company) and 100 mg. of liver extract No. 343 (Eli Lilly and Company).

Inasmuch as it has been suggested by Sure (4) and by others (7) that arachin fails to support normal growth because of the indigestibility of the protein rather than because of an amino acid deficiency, a second basal diet was prepared in which an arachin hydrolysate served as the principal source of dietary nitrogen. The procedure used for the preparation of the arachin hydrolysate was that described by Jackson (8) for making a casein digest. The hydrolysate was incorporated into a basal diet having the following composition: arachin digest 14.5, tryptophane 0.5, Crisco 21, cod liver oil 5, Osborne and Mendel salt mixture (6) 4, and starch 55 per cent. Male rats at weaning were fed this arachin hydrolysate diet *ad libitum*. In addition, each animal received the daily vitamin supplement described above.

*Amino Acid Supplements*—At definite periods during the experiments, the animals received various amino acid preparations as supplements to the basal diet. The amino acids employed were *l*-cystine, *l*-cysteine hydrochloride, *dl*-methionine, and *dl*-homocystine.<sup>1</sup> The growth-stimulating effect of each of these amino acids was tested by incorporation into the basal diets. 360 mg. of *l*-cystine, 470 mg. of *l*-cysteine hydrochloride, 450 mg. of *dl*-methionine, and 402 mg. of *dl*-homocystine were the quantities added singly to each 100 gm. of basal diet when the growth-stimulating action of the respective compounds was being determined.

Throughout the experiment the basal diets and the various supplemented diets were administered to the rats during alternate feeding periods, none of which was less than 3 weeks in length. In this manner each animal was made to serve as its own control.

#### DISCUSSION

Representative graphs illustrating the growth rates of animals under the various conditions of diet are presented in Figs. 1 to 3. Curve A of Fig. 1 demonstrates that arachin is incapable of supporting good growth in animals when it serves as the sole source of protein under the conditions employed. The first portion of Curve A in Fig. 2 shows that even poorer growth was obtained with the basal arachin hydrolysate diet. Additions of cystine to the arachin hydrolysate diet resulted in some stimulation of growth (Fig. 2) but the increase was generally small except in instances in which the animals had been maintained for long periods on the basal arachin hydrolysate diet before cystine supplements were given. It is of interest in this connection to note that during the preparation of the arachin hydrolysate a large portion of the cystine of the arachin was lost when the hydrolysate was neutralized. The sulfur content of the digest was, therefore, considerably reduced. Additions of cystine to the arachin hy-

<sup>1</sup> The *l*-cystine was prepared from hair. The *l*-cysteine hydrochloride was a product of the Eastman Kodak Company and the *dl*-methionine was obtained from Organic Chemical Manufactures Division, University of Illinois. The *dl*-homocystine was prepared from *dl*-methionine by the method of Butz and du Vigneaud (9). All of these compounds were analytically pure.

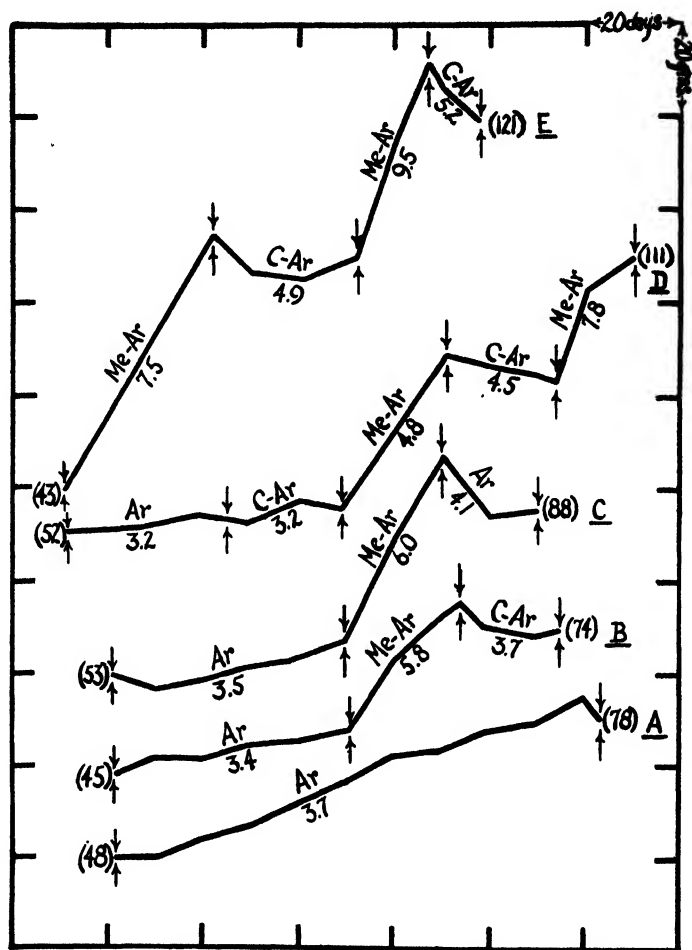


FIG. 1. Growth on basal arachin diet, and on basal arachin diet with added methionine or cystine. The diet employed in any portion of an experiment is indicated by the two downward arrows representing the beginning and end of a period. Diet Ar, basal arachin diet; Diet Me-Ar, 450 mg. of *dl*-methionine per 100 gm. of basal arachin diet; Diet C-Ar, 360 mg. of *l*-cystine per 100 gm. of basal arachin diet. The average daily food consumption in gm. for the corresponding interval is shown by the figures between the upward arrows. The initial and final body weights are presented in parentheses. The cystine, methionine, and basal diet curves are representative of a total of eight, nine, and twenty-nine animals, respectively.

drolysate diet yielded a mixture more closely resembling the basal arachin ration. The growth rates of animals ingesting the ara-

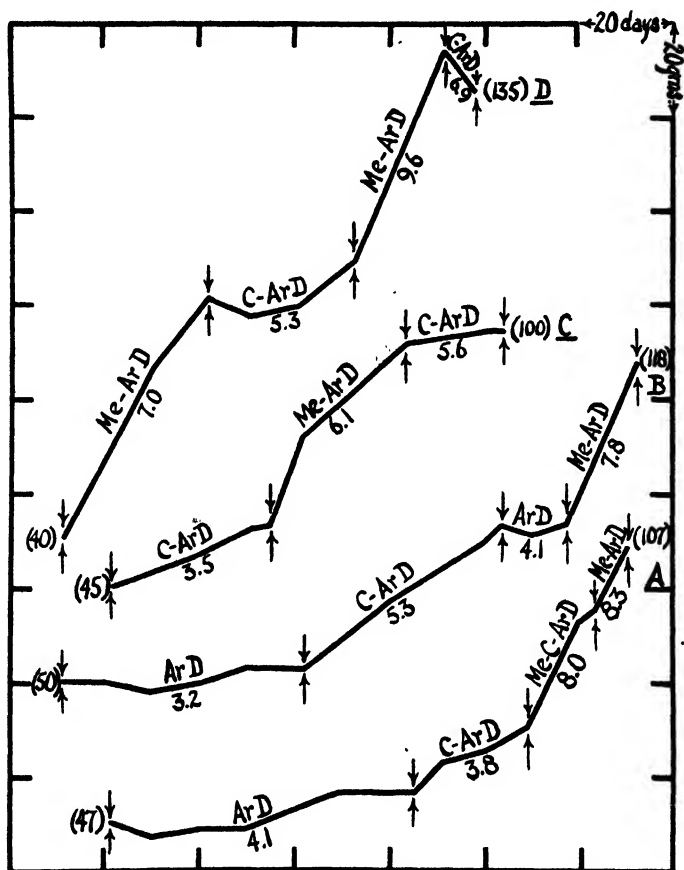


FIG. 2. Growth on basal arachin digest diet, and on this diet supplemented by cystine or by methionine. Diet ArD, basal arachin digest diet. The designations for cystine and methionine supplements, food consumption, and body weights are the same as in Fig. 1. The cystine, methionine, and basal digest diet curves are representative of a total of fourteen, twelve, and ten animals, respectively.

chin hydrolysate diet supplemented with cystine were similar to those of animals ingesting the basal arachin ration (compare Curves A, B, and C, Fig. 2, with Curve A, Fig. 1). It appears,

therefore, that cystine can satisfy a portion of the sulfur requirement in instances in which the sulfur intake is limited. A comparison of the growth rates of animals subsisting on the basal arachin diet and on the basal arachin hydrolysate diet, when the

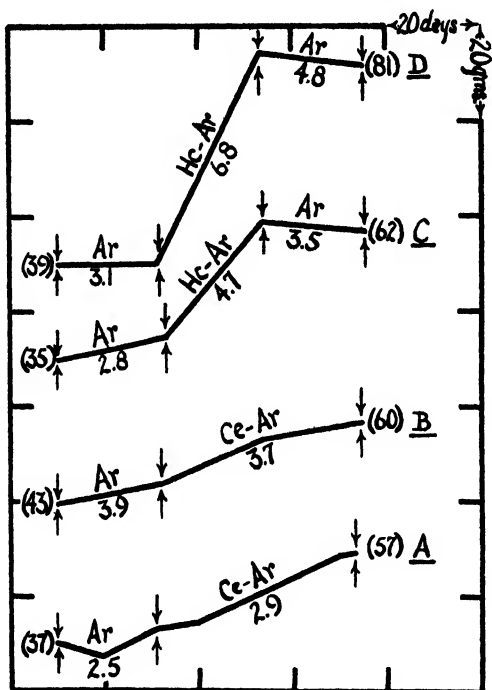


FIG. 3. Growth on basal arachin diet and on basal arachin diet with added *dl*-homocystine or *l*-cysteine hydrochloride. The designations for the basal arachin diet, food consumption, and body weights are the same as in Fig. 1. Diet Hc-Ar, 402 mg. of *dl*-homocystine per 100 gm. of basal arachin diet; Diet Ce-Ar, 470 mg. of *l*-cysteine hydrochloride per 100 gm. of basal arachin diet. The homocystine supplement curves are typical of ten animals and those with cysteine hydrochloride supplements are characteristic of a group of three animals.

latter is supplemented with cystine, demonstrates that the nutritional failure of arachin is probably *not* due to an indigestibility factor.

When the effect of added methionine is observed, it becomes evident that the inability of arachin to support growth is due to

another cause, namely an amino acid deficiency. The addition of this amino acid to either the basal arachin diet (Fig. 1, Curves B, C, D, and E) or the basal arachin hydrolysate diet (Fig. 2, Curves A, B, C, and D) resulted in an immediate, sharp increase in growth rate and a marked rise in the food intake. After periods of supplementing with methionine, a return to the basal diets or to the basal diets supplemented with cystine resulted in cessation of growth or actual decline in body weight. The food intake was correspondingly diminished. The difference in the growth-stimulating ability of cystine and methionine, when used as supplements to the basal diets, proves that these two sulfur-containing amino acids are not completely interchangeable in nutrition as has been hitherto suspected. Although the results do not exclude the possibility that methionine may replace cystine in the diet, they do show definitely that the reverse is not true. Methionine appears to be an essential amino acid in nutrition and the methionine requirement cannot be satisfied by cystine feeding. Similar results have been presented in a preliminary report by Rose and his collaborators (10) which appeared during the course of the present work.

Curves A and B of Fig. 3 show that ingested cysteine hydrochloride, like cystine, cannot replace methionine in nutrition. On the other hand, Curves C and D (Fig. 3) clearly indicate that homocystine, like methionine, is capable of stimulating the growth of rats ingesting the basal arachin diet. The typical rise in food consumption was observed. The response with the homocystine supplement closely resembles that obtained when methionine additions were tested. Apparently homocystine can satisfy a portion, if not all, of the methionine requirement of the albino rat. The results of the experiments suggest that homocystine may function in this respect possibly by virtue of its transformation to methionine by the organism. If the only metabolic pathway of the homocystine supplement were a conversion to cysteine, then homocystine should, like cysteine, be incapable of stimulating growth under the experimental conditions employed. It would seem from the present study, therefore, that when the methionine intake is limited, homocystine may be utilized *per se* or may be converted, by methylation, to methionine.



## SUMMARY

When arachin serves as the chief source of nitrogen in an otherwise nutritionally adequate diet, this protein is incapable of supporting good growth in young rats. The nutritional inadequacy of arachin is attributable to its low methionine content rather than to indigestibility. Cystine, unlike methionine, is incapable of producing growth stimulation when added to the arachin diet. It is therefore evident that methionine is an essential amino acid, quite independent of its relationships to cystine. Homocystine, like methionine, promotes growth when used as a supplement to the basal arachin ration. It seems possible that the ability of homocystine to function in this manner is due to its transformation to methionine by the animal organism.

*Addendum*—After this paper had been presented for publication, an article by Rose (11) appeared which represented a summary of a paper given by him at the joint session of the Federation of American Societies for Experimental Biology at Memphis, April 22, 1937. Professor Rose states, "Contrary to the usual belief, cystine is not an indispensable component of the food. On the other hand, methionine is indispensable." And again, "Of particular interest in this connection is the fact that if methionine is administered at a level which permits maintenance or slow increase in weight, the addition of cystine greatly improves the quality of the diet. Thus, cystine stimulates growth *only when methionine is supplied in sub-optimal quantities*." The results of the present investigation are in complete accord with these observations and coming, as they do, after the lecture of Professor Rose, are now presented as confirmatory evidence, obtained by another mode of experimental approach to the problem of the rôle of the sulfur-containing amino acids in nutrition.

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## DETERMINATION OF DEUTERIUM IN ORGANIC COMPOUNDS\*

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About 2 years ago we described methods for the determination of deuterium in small samples of organic compounds (1). Since that time a number of modifications have been introduced which have simplified the methods and rendered them less liable to error. In principle, the analytical method is still unchanged. The compound to be analyzed is burned, the water formed is purified, and the concentration of  $D_2O$  in this water determined. In practise, however, a very important modification has been introduced: the deuterium content of the finally purified water is determined by two *independent* methods, the agreement of which is taken as the criterion of purity of the water samples. This has been found to be much superior to the earlier method of re-purification of the water and redetermination by the same analytical procedure.

**Combustion**—The sample to be burned is placed in a platinum (or quartz) boat, *b* (Fig. 1). The combustion is carried out in the vitreosil quartz tube, *c*, which is 32 inches long and 1 inch in diameter. One end is drawn down and sealed to a No. 7 interchangeable joint made of quartz, *d*. A trap, *a*, is connected to the quartz tube by the ground joint, and the water formed during the combustion is frozen out in it by means of solid carbon dioxide. The other end of the trap is drawn down to a capillary. To prevent condensation of water in the joint, a collar of sheet copper, *k*, is placed around it and gently heated by a microburner. After

\* This work was carried out with the aid of a grant from the Josiah Macy, Jr., Foundation.

the combustion has been completed and the tube swept out for 20 minutes with oxygen, the capillary is sealed and the other end of the collecting trap closed by a No. 7 ground joint. The combustion tube is loosely packed with CuO for a distance of 18 inches. This section is heated by the furnace to 750°. To compensate for heat losses at the ends of the furnace, auxiliary heating coils, *e* and *f*, are wound directly on the tube. The current in these coils is adjusted so as to keep the temperature of the whole tube at 750°. Just outside the furnace is a 6 inch section of transparent quartz tubing. The boat is placed here so that it can be observed during the combustion. Care should be taken to keep particles of copper oxide from entering the transparent section since this will eventually cause it to become opaque.

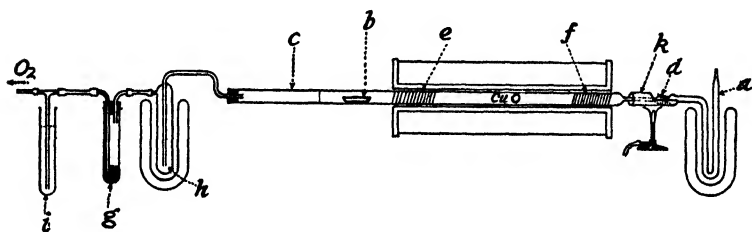


FIG. 1. Combustion apparatus

The oxygen, before entering the combustion tube *c*, bubbles through sulfuric acid in *g* and passes through the trap *h* immersed in solid carbon dioxide. A safety valve, *i*, prevents excess pressure in the system. Oxygen manufactured from liquid air is employed. If electrolytic oxygen is used, a preheater is necessary to remove hydrogen which occurs as an impurity.

Before combustion a blank is taken by running oxygen through the furnace for 30 minutes. No perceptible quantity of ice should collect in trap *a*. Our furnace is heated continuously and a very slow current of oxygen passed through overnight.

According to the procedure published in our previous paper, it was necessary to pass the water thus obtained through a second combustion tube. With the present set-up this step can be eliminated.

For the determination of deuterium in the water of biological

fluids a sample is distilled *in vacuo* from the organ or carcass and about 0.5 cc. is used for analysis. This is placed in the boat or a small glass tube, and the vapor slowly passes through the combustion tube.

*Purification of Water Obtained from the Combustion*—The proper purification of the water samples requires the greatest attention of all the steps in the procedure. In work on intermediary metabolism the deuterium content of organic compounds, and therefore of the combustion water, will in most cases be low. Since the differences between the properties of ordinary water and, for example, 0.1 per cent heavy water are very small,<sup>1</sup> the presence of any impurities may lead to gross errors.

The purification of the water and the analysis should be carried out in a room free of vapors of organic solvents, hydrogen sulfide, etc. The water collected after combustion in trap *a* (Fig. 1) may contain volatile inorganic substances, especially if the compound contained elements other than C, H, and O. Nitrogen-containing compounds give rise to appreciable amounts of oxides of nitrogen, and compounds containing halogens develop free halogen. The nitric acid is removed by the addition of dry barium carbonate and the halogens by copper wire. These modifications became necessary when the analysis of amino acids was undertaken. If halogens are present, a few cm. of carefully cleaned No. 28 copper wire are added to the water in trap *a*. About 12 hours should be allowed for the reaction to go to completion. Excess of carefully dried barium carbonate is now introduced. All the liquid should come in contact with the barium carbonate. It is for this reason that U-tubes are preferable to the traps previously described (1).

The trap *a* is connected with the distillation train (Fig. 2). The entire system, with the exception of trap *e*, is made from 10 mm. glass tubing. All joints except *i* are No. 7 interchangeable. Trap *b* contains a few mg. of dry  $\text{CrO}_3$  and *c* a few mg. of  $\text{KOH}$ <sup>2</sup>

<sup>1</sup> The difference in density between 0.1 per cent heavy water and ordinary water is about 1 part in 10,000, while the difference between their indices of refraction is about 4 parts per million.

<sup>2</sup> The amount of  $\text{KOH}$  used must be kept small since the hydrogen of the  $\text{KOH}$  will lower the deuterium concentration by exchange. Before *a* is attached to the system, the  $\text{KOH}$  is dehydrated by heating *in vacuo*.

and  $\text{KMnO}_4$ . Between the oil pump and the distilling system is placed a trap surrounded by solid carbon dioxide to prevent vapors from the pump from diffusing back into the traps. The system is evacuated and water distilled into the trap *b*, which has been chilled with carbon dioxide. To accelerate the distillation, the trap from which the water is being distilled is immersed in a beaker of water (20–30°). After all the water is collected in *b*, air is admitted through the drying tube *h* and the capillary *g*. The capillary reduces the speed of the air flow so that particles of the drying agent are not swept into the train. A suitable drying agent

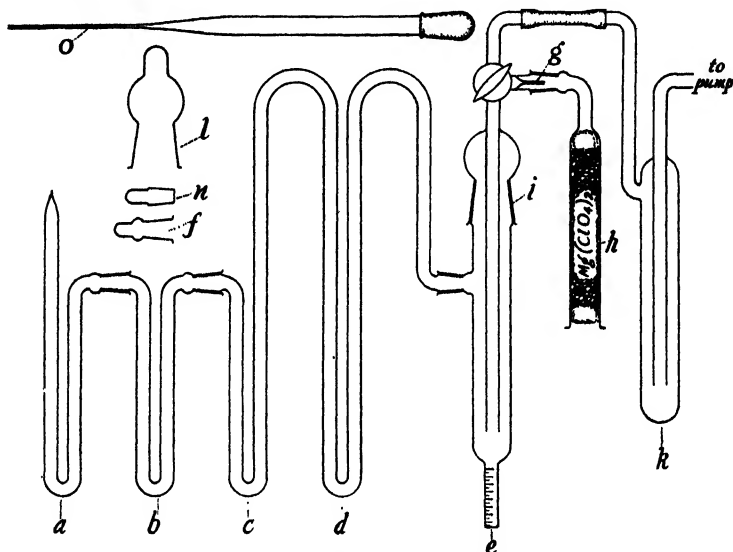


FIG. 2. Distillation apparatus

is  $\text{Mg}(\text{ClO}_4)_2$ . As little as 0.01 mg. of it in 1 cc. of water introduces an observable error in the determination by the interferometer.

The chilling bath is removed from *b*, the ice is melted, and trap *a* is replaced by a No. 7 ground-glass stopper, *f*. The water in *b* is gently boiled by heating with a microburner. Trap *c* is now chilled with carbon dioxide and the water distilled into it *in vacuo*. Air is admitted, the ice is melted, *b* is removed, and the water refluxed. The water is distilled into *d*, melted, and finally collected in *e*. The form chosen for the train is such as to minimize

spraying. The final trap *e* is made of 14 mm. tubing connected at the bottom to an 8 mm. tube calibrated in 0.05 cc. divisions up to 1 cc. The upper end is a No. 15 interchangeable ground joint, *i*. At the end of distillation, *e* is removed from the train and capped with *l* and the side-arm with *n*. If the compound contains only C, H, and O, treatment with  $\text{BaCO}_3$  is unnecessary. Trap *b* can be omitted,  $\text{CrO}_3$  being put into trap *a*.

The sample is now ready to be analyzed. If it amounts to less than 0.4 cc., which is the practical minimum required for analysis by the interferometer, it is diluted with freshly distilled water.

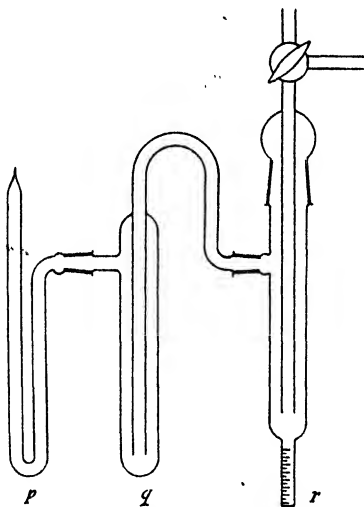


FIG. 3. Redistillation apparatus

For transferring the small samples from trap *e* to the analytical apparatus pipettes *o* (Fig. 2) made from 6 to 8 mm. tubing are employed.

The density and refractive index of the samples are measured. If the deuterium content of the water as determined by these two independent methods differs by more than 0.02 atom per cent, the analysis is rejected and the water redistilled from alkaline permanganate *in vacuo*. The redistillation is performed in the apparatus shown in Fig. 3. If the impurity is suspected to be nitric acid,  $\text{BaCO}_3$  is added.

*Cleaning of Glassware*—All glass with which the water comes in contact must be carefully cleaned. Traps, stoppers, caps, and pipettes are washed with hot solutions of  $\text{Na}_2\text{PO}_4$ . They are rinsed with distilled water, treated with boiling concentrated nitric acid, and then rinsed about ten times with freshly distilled water. They are drained and dried in an electric oven, in which no organic matter (filter paper, towels, corks, etc.) should be placed. It is desirable to use the oven for no other purpose. The final trap (Fig. 2 or 3) is steamed out immediately before use and is dried by means of the vacuum pump.

*Analysis of Water for Deuterium*—The deuterium content of a sample of water is generally determined by measuring the difference in a given property between the sample and ordinary distilled water. As the precise determination of such second order differences is of great difficulty, measurement of two independent properties is advantageous. We rely on the agreement of the values obtained by the measurement of refractive index and density, for impurities in the water generally give rise to errors of different magnitude and sign for these two properties. If only one method is used, a redistillation of the water and redetermination is essential.

*Determination of Refractive Index*—The difference in refractive index between the samples and pure water is determined with a Zeiss interferometer calibrated to read directly in per cent deuterium by means of samples of known deuterium content. These are obtained by diluting a primary standard of about 10 atom per cent deuterium, the density of which has been accurately determined by means of a pycnometer. Before this instrument can be used a table of fringe corrections must be compiled as described by Adams (2) and by Crist, Murphy, and Urey (3). The water used for determination of the zero point of the instrument is distilled directly from alkaline permanganate into the cell. An all-glass system is used. With the 4 cm. cell the deuterium content in 0.4 cc. of water can easily be determined with an error of no more than 0.02 atom per cent. With practise, determinations can be made on 0.3 cc.

*Determination of Density*—Since the density of deuterium oxide is 10.7 per cent greater than that of ordinary water, density determination serves as a convenient method of analysis. Many

methods have been described for the precise determination of the density of water. The methods in general use fall into three classes: pycnometer, submerged float, and the falling drop.

The pycnometer determination of the density of water is usually the primary standard for all the other methods of deuterium analysis. The method is tedious and time-consuming. With extreme care the density can be found to 1 part in a million, corresponding to an error of 0.001 per cent in deuterium. This, however, requires a sample of about 5 cc. The submerged float method is exceeded in precision by no other method. We have used a pressure float in this laboratory for measurements of concentrations below 0.03 atom per cent. Successive determinations can be made to better than 0.0005 atom per cent. However, this method is not readily adapted to routine determinations.

Barbour and Hamilton (4) have described an elegant procedure for the determination of the density of liquids. A drop of fixed volume is allowed to fall through an immiscible liquid of slightly lower density. The velocity of fall is within limits proportional to the difference in density between the drop and the medium. Vogt and Hamilton (5) and Fenger-Eriksen, Krogh, and Ussing (6) applied this method to the determination of the deuterium content of water, and were able to determine the density of water to 1 to 2 parts per million. They used a mixture of bromobenzene and xylene as the immiscible liquid. Since the vapor pressures of the components of this binary system differ, any evaporation changes the composition and density of the surface layer, giving rise to vertical currents. This also changes the calibration. In order to avoid these difficulties we have employed *o*-fluorotoluene as immiscible medium. The middle fraction of the distillate of *o*-fluorotoluene obtained from the Eastman Kodak Company had a relative density of 0.9996 at 26.8°.

The density difference between *o*-fluorotoluene and water can be varied within reasonable limits, since the coefficients of thermal expansion of these two liquids are different. It is thus possible, by adjusting the temperature of the thermostat, to make the falling time of pure water, *i.e.* the zero point, any value compatible with the accuracy desired. We use a 7 c.mm. drop, which in the case of ordinary water falls 15 cm. in 180 seconds. A difference in falling time of 1 second corresponds to about 2.5 parts per mil-



lion in the density in this range (0.002 per cent deuterium). A sample of 0.10 per cent  $D_2O$  falls in 140 seconds. With drops falling in about 180 seconds it is possible to compare two water samples to 1 part per million in density.

**Tube and Thermostat**—The tube holding the *o*-fluorotoluene is 1 cm. in inside diameter, 55 cm. long, and has two circles engraved on it 8 and 23 cm. from the bottom, respectively. It is sealed at the bottom and closed at the top with a No. 11 ground glass joint. The tube is filled with *o*-fluorotoluene to within 5 cm. of the top and is immersed in a thermostat to within 3 cm. of the top. The tube should be rigidly supported so that the stirring of the bath does not cause it to vibrate, and great care must be taken that the axis of the tube is vertical.

The thermostat is a glass tank of 100 liters capacity; the temperature is regulated to  $0.001^\circ$ . Two strips of the thermal insulation are removed so that the tube can be observed. The time of fall of the drop between the two marks is determined with a 0.1 second stop-watch.

**Micropipette**<sup>3</sup>—A drop of uniform volume is secured by the micropipette shown in Fig. 4. The screw *A* when turned forces down the piston *P*, which is loosely fitted in the hole in plate *D*. The gasket *G* is made of a good quality gum rubber. The size of the drop is determined by the angle between the stops *S*<sub>1</sub> and *S*<sub>2</sub>. The glass portion of the apparatus is attached to the steel plate *D* by de Khotinsky cement. Capillary tubing (2 mm.) is used to minimize the volume of the pipette. The end of the pipette, *H*, is drawn to a fine tip. The entire pipette is moved up and down by means of the rack and pinion.

The pipette is completely filled with mercury. The best results are obtained when there is no air trapped in the pipette. This can be accomplished by filling it *in vacuo*. The pipette is turned upside down, evacuated through *H*, and the mercury allowed to flow in through *K*. The presence of air in the pipette can readily be determined by rotating the whole pipette about an axis perpendicular to the plane of the illustration. Under air-free conditions the mercury meniscus at *H* will not move.

**Operation of Pipette**—A sample of the water, the density of

<sup>3</sup> The authors are highly indebted to Mr. F. Rosebury for valuable assistance in the construction of the micropipette.

which is to be determined, is placed in a small quartz tube. The tip *H* is immersed below the surface of the water. The stop-cock *F* is opened and a drop of mercury allowed to flow out. Suction is applied at *K* and the water drawn up into the tip *H* to flush it out. Another drop of mercury is now allowed to flow out and suction is again applied at *K* to bring the water above the point where the tube *H* has been drawn down to form a tip. The screw

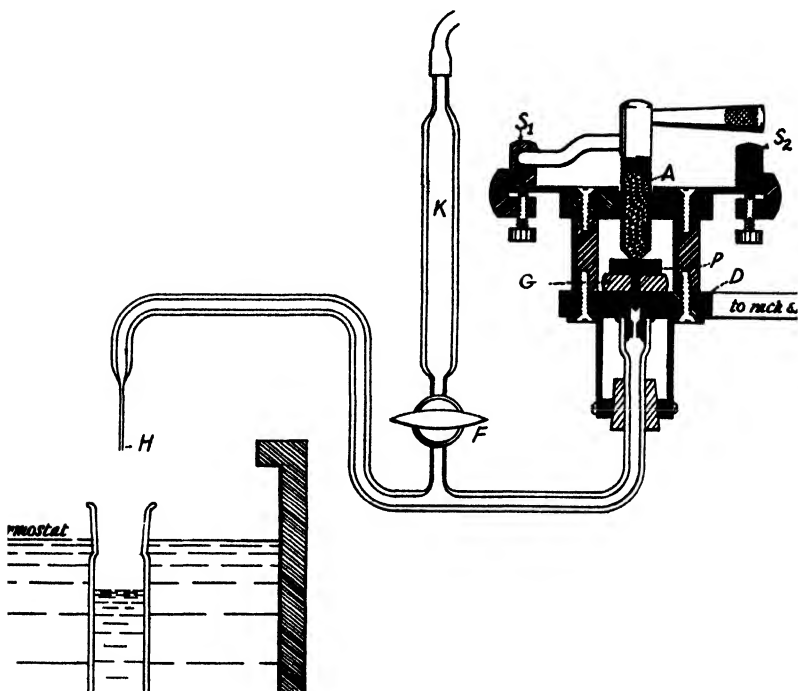


FIG 4 Micropipette

*A* is now turned from *S*<sub>1</sub> to *S*<sub>2</sub> to fill the pipette and the tip is wiped with hard filter paper. The pipette is lowered by the rack and pinion until *H* is below the surface of the *o*-fluorotoluene. When the tip of the pipette is thus immersed, the water may be observed as a dark line in the constricted portion of the capillary. One should always be certain that the dark line extends to the end of the tip before squeezing out the drop. The screw *A* is now

rotated from  $S_2$  to  $S_1$ , expelling a drop of water. The pipette is raised slowly and, as the tip passes through the surface, the drop is detached.

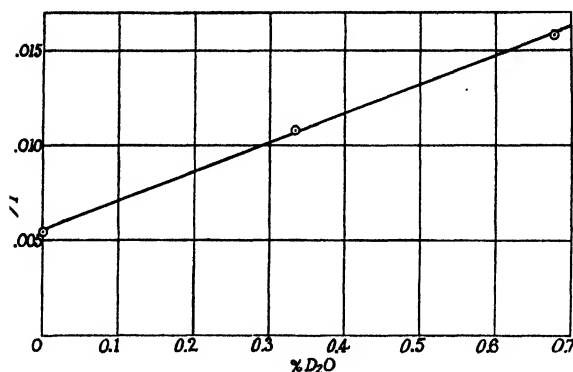


FIG. 5. Calibration curve for falling drop method (low concentrations of  $D_2O$ ).

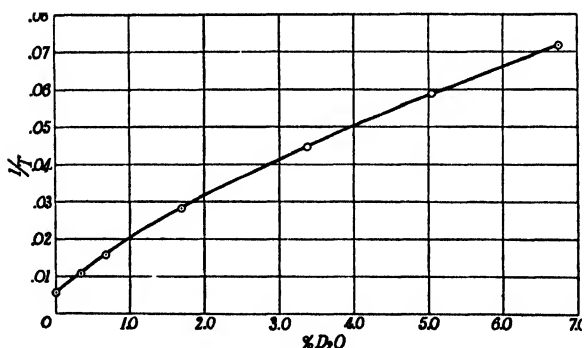


FIG. 6. Calibration curve for falling drop method (high concentrations of  $D_2O$ ).

The velocity of the fall of the drop is not a linear function of the density of the water, although between 0.0 and 0.7 atom per cent deuterium (the difference in density between medium and sample ranging from 375 to 1100 parts per million) the deviation from linearity corresponds to less than 0.01 atom per cent deuterium.

Typical calibration curves for low and higher concentrations are shown in Figs. 5 and 6. In these graphs are plotted the reciprocal of the falling time against per cent  $D_2O$ . The great advantage of using *o*-fluorotoluene is that the calibration curve does not change from day to day.

#### SUMMARY

Different methods are described for the determination of deuterium in small amounts of organic compounds.

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# AN ULTRACENTRIFUGAL STUDY OF THE pH STABILITY OF TOBACCO MOSAIC VIRUS PROTEIN

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## PLATES 1 AND 2

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It has been shown by Svedberg and his pupils that much valuable information about protein molecules can be gained by studying their ultracentrifugal patterns (1) at various pH values. In the case of the virus proteins and other macromolecular substances suspected of having specific biological activities, such a determination of the pH range of molecular stability takes on an added importance, for, if it is found that the heavy molecules are stable within the same limits that define the biological activity, then evidence has been obtained that the activity is really associated with the molecules being studied. Such a comparison has been made for infectious papillomatosis (Shope) in rabbits (2). The present paper describes the results of an ultracentrifugal analysis of the pH stability of tobacco mosaic virus protein; comparisons can be made between these results and determinations of the pH range of activity of this virus (3-5).

The writer is indebted to W. M. Stanley of this Institute for the virus protein samples used in this investigation and for many of the determinations of pH. In order to avoid the chemical alterations that occur when tobacco mosaic virus proteins are subjected to the prolonged action of concentrated salt solutions, these samples were extracted and purified from the juice of diseased Turkish tobacco plants by quantity ultracentrifugation. The apparatus used for this purpose has already been described (6). The first experiments were made upon a protein (No. III, 2B) derived from plants harvested 3 weeks after inoculation. This protein, which was prepared with 0.1 M neutral phosphate

buffer as solvent, had stood for some time in the ice box before use and was double boundaried (Fig. 9). Previous work (7) had shown that such a protein should be single boundaried (Fig. 1) when freshly prepared. Studies, accordingly, were made with a fresh sample (Protein III, 1B) to ascertain whether any differences could be observed between otherwise similar single and double boundaried proteins. At about this time it was discovered that the second molecular component arises through contact of the unaltered protein with salt and that when distilled water is used as solvent, the purified protein is and remains single boundaried. In view of this evidence (7) that even 0.1 M phosphate may bring about molecular changes in some native tobacco mosaic virus proteins, trials were made on a third water-extracted preparation to see whether it differed appreciably in its pH reactions from the salt-extracted samples. This water-extracted protein (No. II, W) was obtained from plants cut 2 weeks after inoculation.

The ultracentrifuge arranged for absorption measurements and the photographic procedures were those employed in the preceding study (7). The optimum concentration of tobacco mosaic protein for the 2 mm. thick absorption cell that has been used is about 1.5 mg. per cc. The three proteins studied were made up in solutions containing 3 to 6 mg. per cc.; the first two were dialyzed against running water to remove all but a trace of the salt they contained. Samples to be analyzed in the ultracentrifuge were prepared by mixing appropriate amounts of these proteins with about 0.2 M salt buffers of various pH's between 1.5 and about 11. The pH values of the buffers and of resulting mixtures were read with one or the other of two closely agreeing glass electrodes. Ultracentrifugal photographs of the samples were made immediately after mixing and after different intervals of time up to 2 months. When computed in the usual fashion, measurements on these photographs have yielded the sedimentation constants collected in Table I. For the present purposes it has been considered sufficient to employ photographs made during the routine operation of the centrifuge without taking all the precautions that are needed for the highest accuracy. The variation in the duplicate results of Table I indicates the kind of agreement that can easily be obtained in this fashion. While any molecular changes observed in the photographs taken immediately

TABLE I

*Sedimentation Constants of Tobacco Mosaic Virus Proteins at Different pH Values*

U. indicates the presence of light unsedimentable material; N.U. its absence. In many experiments the air bubble needed for this observation was missing. The length of time, in hours or days, that a solution had stood at a stated pH is indicated in parentheses following the sedimentation constant; absence of parentheses means that the photograph was made immediately after pH adjustment. When most of the protein was in a rapidly sedimenting colloidal suspension rather than in true solution, (col.) has been added; (dif.) indicates a very diffuse boundary.

pH	Protein III, 2B	Protein III, 1B	Protein II, W
1.5	U. 179, 220, 244 " (12 hrs.)		
1.8	N.U. 174 " 169 (18 hrs.) 173, 218 (3 days) N.U. 169, 207 (6 days)	179 N.U. 169, 209 (3 days)	173 212 (65 days)
2.8	N.U. 184 (col.)	Insoluble	Insoluble
4.0	Insoluble		
5.0	N.U. 186, 210	N.U. 238 (col., dif.)	
6.1	184, 210	224 (dif.) 179, 209 (10 days)	174, 210 (dif.) 247 (65 days; col., dif.) 178 176 (1 day) 176 (3 days)
Water dilutions			
7.0	178, 206 169, 189	N.U. 172 175, 201 (9 days)	173 172, 206 (2 days) 184, 209 (63 days)
8.0	172, 201 167, 200 172, 218 (1 day) 171, 206 (3 days)	170 N.U. 173 171, 206 (5 days)	169 177, 200 (64 days)
9.0	168, 199 151, 191 (2 days)		177 151, 185 (2 days) 143, 185 (4 " ) U. 157, 181 (66 days)
10.0		166 89, 139, 178 (16 hrs.) U. 80, 144, 182 (3 days)	N.U. 180 86, 130, 174 (1 day) 99, 149, 181 (3 days) U. 129, 179 (66 " )
11.0	N.U. 47, 164 59, 175 50, 159 U. 19, 73, 165 (18 hrs.) U. 24, 184 (6 days)		



after pH adjustment may safely be ascribed to pH, those seen in photographs made after standing are due to the combined action of pH and about 0.1 M salt.

Working with chemically prepared protein, Eriksson-Quensel and Svedberg (8, 9) found that splitting of the molecules had taken place at pH 9.8, yielding molecular components with  $s_{20}^{\circ} = 185$  and 125; at pH 11.7 the fragments had become small with  $s_{20}^{\circ} = 8.1$  and 3.8. In so far as they can be compared, these results are in essential accord with those of the present study.

Typical photographs made immediately after preparing the samples are shown in Figs. 1 to 17. Between pH about 2.8 and pH about 5, in the neighborhood of the isoelectric point, the proteins are so insoluble that no sedimentation picture can be obtained. On the more alkaline side of the isoelectric point up to pH about 10 the sedimentation constants of the molecular components found in solutions immediately after pH adjustment are those of the unaltered virus protein molecules. At pH 11 the protein is split, giving much "unsedimentable" material<sup>1</sup> as well as two fairly homogeneous substances with apparent constants  $s_{20}^{\circ} =$  about 50 and about  $170 \times 10^{-13}$  cm. sec.<sup>-1</sup> dynes<sup>-1</sup>. The lighter of these is obviously a degradation product; the heavier is probably not very different in molecular size from the original virus protein.

When the protein redissolves on the acid side of its isoelectric point, originally double boundaried as well as single boundaried material has at first only one molecular component (Fig. 3) which sediments at the same rate as the principal component found in neutral and weakly alkaline solutions. An immediate change in the molecular composition of acid protein solutions occurs at a point between pH 1.8 and 1.5.

The pH range of molecular stability is more restricted when one considers the photographs made of solutions which have stood for various lengths of time at these different pH values. After 2 days, and probably sooner, unsedimentable material is present at pH 9 (Fig. 13) and the two boundaries that are observed come

<sup>1</sup> Because of the viscosity of this unsedimentable material and of the other split-products of the virus protein, no great accuracy can be ascribed to the sedimentation constants measured (Table I) for any of these inactive strongly alkaline solutions.

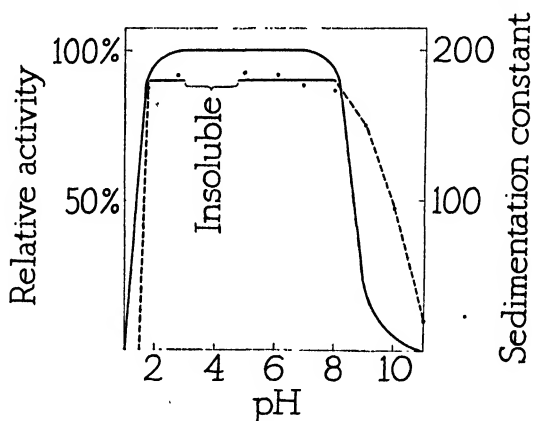
down less rapidly than those in neutral solution; isolation with the quantity centrifuge and measurements on the solutions thus purified would be needed to show whether or not these boundaries are due to molecules appreciably lighter than those of the active protein. Photographs of the still more alkaline solutions of pH 11 after 18 or more hours show a further degradation of the molecules present; a new light component with an apparent  $s_{20}^0 =$  about 20 has appeared and small amounts of other transient molecular fragments seem to be present. On the acid side observations at pH 1.8 indicate that the molecularly homogeneous protein gradually becomes double boundaried (Fig. 4) with the same sedimentation constants that were observed in the neutral protein samples. Only unsedimentable material could be seen in a solution of pH 1.5 after 12 hours.

Studies with the two single boundaried virus protein samples (Proteins III, 1B and II, W) offer a general confirmation of the results with Protein III, 2B. In neutral and weakly alkaline solutions they become double boundaried in 0.1 M buffer, as would be expected. At pH 10 they are at first single boundaried (Fig. 15), but they show unsedimentable material and develop boundaries due to rather large products with apparent constants of about 85, 140, and 175 (Figs. 16 and 17). The photographs at pH between 9 and 11 suggest that the tobacco mosaic virus protein can split in a number of steps or ways, and that the mean size of the resulting fragments is smaller in a more alkaline medium. Many of these fragments appear stable enough for isolation by the methods of quantity ultracentrifugation. When this has been done, accurate measurements of their true sedimentation constants and molecular weights can be made and useful information can thus be obtained concerning the internal structure of the virus protein molecule itself.

Clearly defined differences among the three proteins are apparent near their isoelectric points. On the acid side, at pH 2.8, a faint boundary can be measured for the double boundaried sample but none for either Protein III, 1B or Protein II, W. At pH 5 all of Protein III, 2B is in solution (Fig. 7), while single boundaried material gives only faint and heavy boundaries (Fig. 8); even at pH 6 good boundaries are obtained only from Protein III, 2B (Figs. 9 and 10). These results indicate that the native

single boundaried proteins remain out of solution over an appreciably wider pH range about the isoelectric point than do proteins that have been altered by contact with salts. It is not established whether the boundary diffuseness and frequent high sedimentation constants of the single boundaried proteins at pH 5 and 6 are merely consequences of this lower solubility or whether a decreased molecular stability also plays a part.

The circumstances under which the infectivity tests (3-5) and the present experiments have been made are not identical, but in so far as their results are comparable they show that the



TEXT-FIG. 1. A composite plot of the dependence of pH of the virus activity (full curve, taken from (10)) and of the sedimentation constants of the principal molecular component in virus protein solutions (dotted curve).

pH ranges of molecular stability and of infectiousness parallel one another. This can be seen by comparing the infectivity curves (4) with the data of Table I; it is shown graphically in Text-fig. 1, which is a composite plot of the way the virus activity (4, 10) and the sedimentation constants of the principal molecular component depend on pH. Both the infectivity and the molecules producing the boundaries seen in the virus protein solutions remain unaltered for at least a day in the pH range from about 2 to about 8. On the acid and alkaline sides of these limits the infectivity falls rapidly. In alkali it does not immediately reach zero but instead seems to become stabilized (4) at a reduced

level. Some molecules about the size of those of the original virus protein still exist in these alkaline solutions, but detailed studies are needed to show whether they are responsible for the infectivity that remains.

#### SUMMARY

An ultracentrifugal study has been made of the molecular stability of the tobacco mosaic virus protein in solutions at a series of pH values between 1.5 and 11. On the alkaline side of the isoelectric point the molecules of the protein remain unchanged for at least 2 months at pH up to 8. Decomposition has occurred at pH 9; it is more rapid as the pH is raised and is practically instantaneous at pH 11. The split-products are smaller in the more alkaline solutions. On the acid side molecular changes begin between pH 1.8 and 1.5; at pH 1.5 destruction is complete within a few hours.

The three virus protein samples used in these experiments were isolated by quantity ultracentrifugation and gave sharply sedimenting boundaries. One was double boundaried; the other two contained only one molecular component. Differences between these proteins were observed at pH values near the isoelectric point.

In so far as different experimental conditions permit comparisons to be made, the effects of pH on infectivity and molecular stability are parallel. This agrees with the assumption that infectivity is a property of the protein molecules.

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## EXPLANATION OF PLATES

## PLATE 1

FIG. 1. The sedimentation diagram of a water solution of the virus Protein II, W. The high degree of molecular homogeneity of this protein is shown by the perfect sharpness of the boundary even in the last photograph of the series. Mean centrifugal field = 23,300 times gravity. In this and the following figures the individual pictures of a series were made at intervals of exactly 5 minutes with light of wave-lengths between about 2700 Å. and about 2300 Å.

FIG. 2. The sedimentation diagram of Protein III, 2B after 1 hour at pH 1.5. The boundaries have become diffuse and some unsedimentable material is already present. The most conspicuous boundary corresponds to the sedimentation constant 220 (Table I). Field = 24,100 times gravity.

FIG. 3. Sedimentation photographs of Protein III, 2B in buffer of pH 1.8 immediately after mixing. Note the single boundary. Field = 11,400 times gravity.

FIG. 4. Sedimentation photographs of Protein III, 2B in buffer of pH 1.8 after 6 days. The single sharp boundary of Fig. 3 has been replaced by two more diffuse ones. Field = 11,200 times gravity.

FIG. 5. Sedimentation photographs of Protein II, W in buffer of pH 1.8 immediately after mixing. The single boundary is slightly diffuse. Field = 23,900 times gravity.

FIG. 6. Sedimentation photographs of Protein II, W in buffer of pH 1.8 after 65 days. The boundary has become so diffuse that its exact position cannot be seen in the later pictures. Field = 23,500 times gravity.

FIG. 7. Sedimentation diagram of Protein III, 2B in buffer of pH 5 immediately after mixing. Two boundaries can be seen in the original negative. Field = 11,400 times gravity.

FIG. 8. Sedimentation diagram of Protein III, 1B in buffer of pH 5 immediately after mixing. Most of the protein is in heavy colloidal suspension, but the faint boundaries due to the small amount remaining in solution are indicated by arrows in the third and fourth pictures. Field = 11,600 times gravity.

## PLATE 2

FIG. 9. Sedimentation photographs of Protein III, 2B in buffer of pH 6 immediately after mixing. Field = 11,500 times gravity.

FIG. 10. Sedimentation photographs of Protein III, 1B in buffer of pH 6 immediately after mixing. The diffuse boundary of this figure is to be contrasted with the two sharper boundaries of Fig. 9. Field = 12,100 times gravity.

FIG. 11. Sedimentation diagram of Protein II, W in buffer of pH 6 immediately after mixing. The faint sharp boundary indicated by the arrows has the same sedimentation constant ( $s_{20}^0 = 174$ ) as the original protein, but most of the absorption is in the heavier diffuse boundary. Field = 24,000 times gravity.

FIG. 12. Sedimentation diagram of Protein II, W in buffer of pH 6 after 65 days. Most of the absorbing material is in colloidal suspension; the boundary marked by an arrow in the second exposure sediments at a faster rate than the heavier diffuse one of Fig. 11. Field = 23,500 times gravity.

FIG. 13. Sedimentation diagram of Protein II, W in buffer of pH 9 after 4 days. The boundary marked (b) is as sharp as that of the original protein; (a) is more diffuse. Field = 23,500 times gravity.

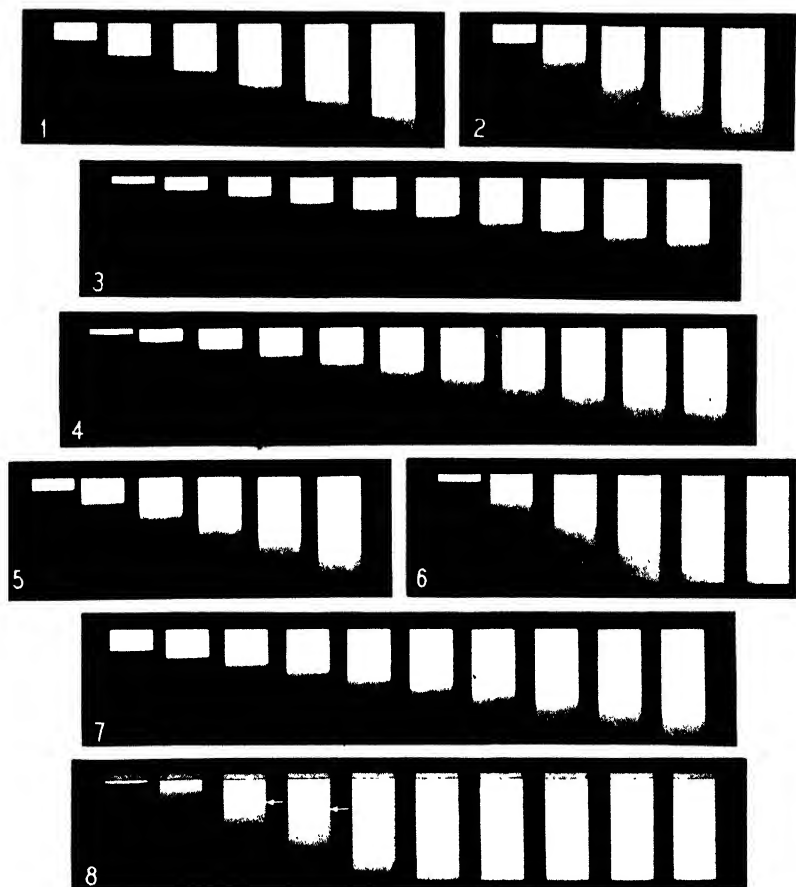
FIG. 14. Sedimentation diagram of Protein II, W in buffer of pH 9 after 66 days. The boundaries are somewhat less sharp than in Fig. 13. Note the unsedimentable material. Field = 24,000 times gravity.

FIG. 15. Sedimentation photographs of Protein II, W in buffer of pH 10 immediately after mixing. The protein is not yet measurably altered. Field = 24,200 times gravity.

FIG. 16. Sedimentation photographs of Protein II, W in buffer of pH 10 after 1 day. The boundaries marked (c), (d), and (e) are due to components with apparent constants of about 90, 140, and 175. Field = 23,600 times gravity.

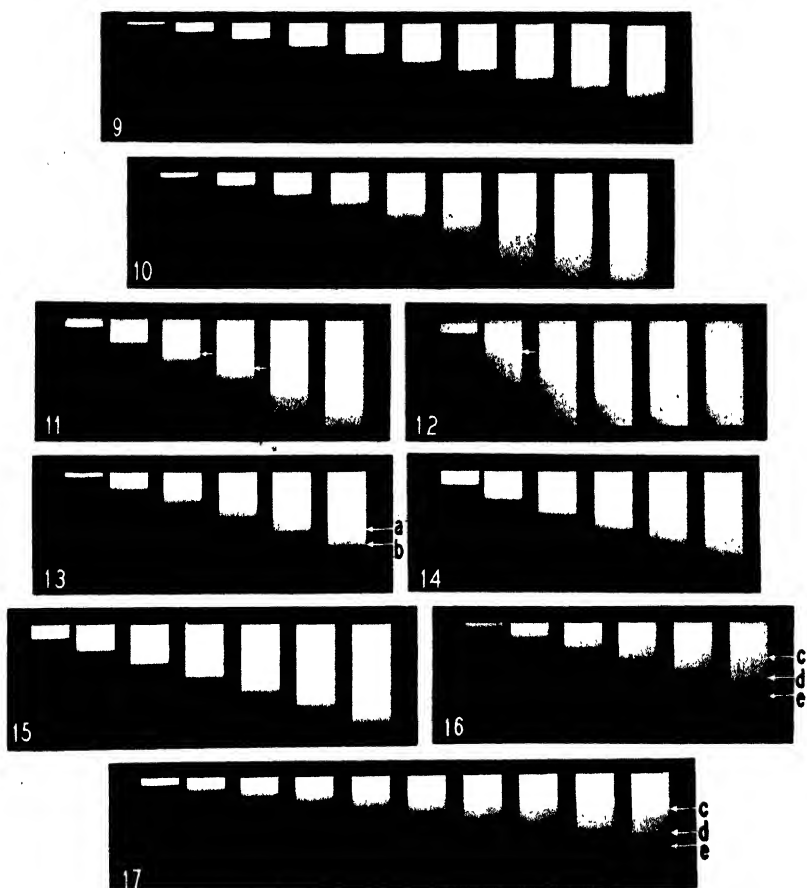
FIG. 17. Sedimentation photographs of Protein III, 1B in buffer of pH 10 after 3 days. Compared with Fig. 16 it is seen that the lightest component (c) has increased at the expense of the other two. Note also the unsedimentable material. Field = 12,500 times gravity.













# **METABOLISM AND MODE OF ACTION OF VITAMIN D**

## **IV. IMPORTANCE OF BILE IN THE ABSORPTION AND EXCRETION OF VITAMIN D**

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(Received for publication, October 9, 1937)

The rôle of bile in the intestinal absorption of vitamin D has been studied by Greaves and Schmidt (1), but no conclusive investigations have dealt with its importance in the excretion of vitamin D. The reinvestigation of the rôle played by bile in intestinal absorption of vitamin D that is included in the scope of the studies presented here seemed advisable for the following reason. The use of crystalline vitamin D<sub>2</sub> dissolved in propylene glycol (drisdol), as compared with the use of viosterol in oil, makes it possible to dissociate the influence of the solvent on absorption of vitamin D. Because of results recently obtained (2), it furthermore seemed advisable not to depend on incurability of rickets as the criterion of failure in absorption of vitamin D in animals with liver injury, but rather to detect the presence of vitamin D in the blood serum of these animals after they had been fed vitamin D by mouth in the form of viosterol in oil or of drisdol.

### **EXPERIMENTAL**

Ten dogs weighing 6 to 12 kilos were given food devoid of vitamin D. They were kept in metabolism cages in a dark room. In order to prevent contamination of the food with feces, the cages were cleaned twice daily and the dogs were fed outside the cages.

Double ligation and transection of the common bile duct in its lower third portion were performed under ether or sodium pentobarbital anesthesia. Biliary fistula was established by inserting a glass cannula into the gallbladder after the common duct had been ligated. The cannula was fastened with a purse-string suture in the gallbladder, and the omentum was stitched near the

cannula to the gallbladder wall. A U-shaped rubber tube was attached to the cannula, through which bile drained out of the left side of the abdomen. It was thus possible to collect bile under sterile conditions in a football bladder in the usual manner.

Vitamin D was administered as viosterol in oil or as drisdol, in a dose of 20 to 25 cc.,<sup>1</sup> by stomach tube or by intramuscular injection into the thigh, half of the dose being given in each side. The time interval between operation and administration of vitamin D varied from dog to dog and is shown in each instance in Tables I to III. Blood was taken always after 18 to 20 hours of fasting.

### *Method*

The presence of vitamin D in the blood serum of the dogs was detected by injecting daily 0.3 to 0.5 cc. of the serum intramuscularly into rats for 10 consecutive days. The rats had been fed Steenbock's rachitogenic Ration 2965<sup>2</sup> since the age of 3 weeks, for 3 consecutive weeks. Healing of rickets was checked by roentgenograms taken just before treatment was started as well as 8 to 10 days after. Content of phosphorus and calcium in the serum of the dogs and of the rats was determined by Samson's (3) titrimetric method.

The presence of vitamin D in the bile was detected by feeding bile extracts to rachitic rats under the conditions described. The extracts were prepared according to a procedure reported previously (4), the only deviation being that whenever the amount of bile collected in the bile fistula dogs exceeded 80 cc. the residue was dissolved in an amount of oil equal to one-twelfth of the quantity of bile used, instead of one-sixth as in the earlier method.

Each extract was tested on two to three rats. The dog serum, however, could not be obtained in amounts large enough to allow its administration to more than one rat at a time; frequent determinations of vitamin D served to protect against possible errors.

### *Results*

*Intestinal Absorption of Vitamin D*—Viosterol in oil or drisdol was given by stomach tube to two control dogs and to three dogs

<sup>1</sup> 200,000 to 250,000 U.S.P. units of vitamin D.

<sup>2</sup> Steenbock, H., and Black, A., *J. Biol. Chem.*, **64**, 274 (1925).

in which the common bile duct had been ligated and transected 5 days to 3 weeks previously. Whether or not the vitamin D had been absorbed\* was shown (1) by presence or absence of vitamin D in the blood serum and (2) by a hyperphosphatemic reaction or the lack of such a reaction following administration of vitamin D.

After the vitamin D preparation had been given by mouth to the two control dogs, the antirachitic efficacy of the blood serum, which was not antirachitic or was only slightly so before the administration of viosterol or drisdol, increased considerably (Table I). As had been the case with results obtained with rabbits (5), vitamin D was found to be circulating in the blood of these dogs for an impressive length of time—for 10 or 11 months when administered in the form of viosterol and for from 1 to 4 months when given as drisdol.

The additional observation that there was a definite increase in inorganic phosphates in the blood of both dogs after administration of vitamin D left no doubt that the vitamin D had been absorbed in these animals.

The results of giving the same doses of viosterol in oil or of crystalline vitamin D dissolved in propylene glycol by stomach tube to the three dogs in which the common bile duct had been ligated are clear cut. No vitamin D could be detected in the blood serum and the hyperphosphatemia observed in the controls was not present.

The results of these experiments confirm the conclusion of Greaves and Schmidt and prove, beyond doubt, that bile is essential for the intestinal absorption of vitamin D. The conception that interference with absorption of oil in the absence of bile would explain failure in absorption of vitamin D given in the form of viosterol in oil can be discarded, in view of the finding that vitamin D is not absorbed even when it is dissolved in propylene glycol. The conclusion can therefore be drawn that the presence of bile is essential for the intestinal absorption of the vitamin molecule.

*Excretion of Vitamin D*—In order to learn whether or not bile excretes vitamin D, viosterol and drisdol were given by intramuscular injection to six dogs in which the common bile duct had been ligated. The presence of vitamin D in the bile of these animals was detected by feeding bile extracts to rachitic rats in the manner described. Because the accumulated bile was ob-

TABLE I  
*Intestinal Absorption of Vitamin D Administered As Viosterol or Drisdol by Stomach Tube to Dogs with and without Obstruction of Common Bile Duct*  
 0 indicates no healing, (+) trace, + slight, ++ moderate, +++ almost complete, ++++ complete.

Dogs with ligation and transection of common bile duct										Dogs with ligation and transection of common bile duct										
Dog	Date	Weight	Blood serum of dogs		Vitamin D in dog serum* shown by degree of healing in x-rays of rats		Dog	Date	Weight	Blood serum of dogs		Vitamin D in dog serum* shown by degree of healing in x-rays of rats		Dog	Date	Weight	Blood serum of dogs		Vitamin D in dog serum* shown by degree of healing in x-rays of rats	
			Phos-phorus mg. per 100 cc.	Cal-cium mg. per 100 cc.	After 8 days	After 10 days				Phos-phorus mg. per 100 cc.	Cal-cium mg. per 100 cc.	After 8 days	After 10 days				Phos-phorus mg. per 100 cc.	Cal-cium mg. per 100 cc.	After 8 days	After 10 days
A	Mar. 31, 1936	6.7			0	(+)	B	Mar. 31, 1936	3.5	10.03	14.6			C	Mar. 31, 1936	3.5	10.03	14.6	0	+
	Apr. 6, 1936	6.8	7.32	13.4	0	+		Apr. 6, 1936	3.8	9.75	14.0				Apr. 6, 1936	3.8	9.75	14.0	0	(+)
	" 13, 1936		7.43	13.8				" 13, 1936		8.26	13.2				" 13, 1936		8.26	13.2		
	25 cc. viosterol in oil given							" 15, 1936							" 15, 1936					
	Apr. 15, 1936	7.6	9.04	13.8	++	++		" 18, 1936							" 18, 1936		7.99	13.4		Operated
	" 18, 1936		7.33	14.0	++	+++		" 20, 1936		6.94	11.2				" 20, 1936		6.94	11.2	0	0
	" 22, 1936		6.82	13.8				20 cc. viosterol in oil given							Apr. 22, 1936		4.21	10.9	0	0
	" 27, 1936	8.2	8.16	13.6	++	++		" 24, 1936							" 24, 1936		6.10	11.6	0	0
	May 6, 1936		6.55	13.8	++	++		" 27, 1936	3.4	6.55	11.6				" 27, 1936	3.4	6.55	11.6	0	0
	" 13, 1936	8.8	7.6	14.0	++	++		" 29, 1936		6.25	12.4				" 29, 1936		6.25	12.4		
	" 27, 1936		7.2	10.4	++	++	C	May 4, 1936		5.2	14.2				May 4, 1936		5.2	14.2		
	June 10, 1936	10.4	8.7	13.2	++	++		" 11, 1936	2.8	6.5	14.8				" 11, 1936	2.8	6.5	14.8	0	0
	" 24, 1936			15.8	++	++		Aug. 19, 1936	10.0	5.1	12.4				Aug. 19, 1936	10.0	5.1	12.4	+	++
	July 7, 1936	11.0			++	++		" 28, 1936	10.4	6.9	11.8				" 28, 1936	10.4	6.9	11.8	+	++
	" 21, 1936	11.4			++	++		Sept. 3, 1936	10.4	6.3	11.6				Sept. 3, 1936	10.4	6.3	11.6		Operated
	Aug. 4, 1936				++	++		" 5, 1936		6.43	12.6				" 5, 1936		6.43	12.6		
	" 19, 1936	12.4	5.4	13.6	+	+		" 14, 1936	9.6	6.6	11.0				" 14, 1936	9.6	6.6	11.0		
	Sept. 3, 1936	12.0	6.9	12.0	+	+		" 18, 1936		4.7	11.0				" 18, 1936		4.7	11.0		
	" 14, 1936	12.5	6.15	12.4	(+)	(+)		" 21, 1936		6.3	11.4				" 21, 1936		6.3	11.4		
	Oct. 14, 1936	12.0	3.5	13.1	+	+														

[illegible]

\* From Dog A 0.5 cc. and from Dogs H, B, C, D 0.3 cc. of serum were injected into the rachitic rats daily.



tained *post mortem* by aspiration of the distended gallbladder, it was felt that the results (Table II) might justly be subject to criticism. They were therefore checked in two dogs with fistula of the gallbladder; bile was collected quantitatively *intra vitam* without having been subjected to stasis (Table III). The data in Tables II and III show that in bile are excreted only small and rather negligible amounts of vitamin D injected as viosterol in oil but rather large amounts when drisdol is given. The chemical nature of the solvent, consequently, has a definite influence on the excretion of vitamin D from liver tissue into bile. Further studies will

TABLE II

*Excretion of Vitamin D in Bile of Six Dogs with Ligation and Transection of Common Bile Duct after Intramuscular Injection of Vitamin D*

0 indicates no healing, (+) trace, + slight, ++ moderate, +++ almost complete.

Dog	Vitamin D injected		Time between injection of vitamin D and death of dog*	Vitamin D in dog bile shown by degree of healing in x-rays of rachitic rats fed bile extracts	
	Preparation	Amount		For 8 days	For 10 days
		cc.			
B	Viosterol in oil	20	6 wks.	0, 0	0, 0
D	" " "	20	1½ days	0	0
M	" " "	25	5 "	0, +	(+), +
C	Drisdol	25	2 wks.	++, ++, +++	+++, ++, +++
G	"	25	2 "	+, +	+, ++
L	"	25	5 days	+, ++	++, +++

\* Operation preceded injection by 2 to 4 days.

have to be made to show whether or not this effect is due to an increased retention of vitamin D in the liver when vitamin D is given in oil.

#### SUMMARY

1. Vitamin D was not present nor was there a hyperphosphatemic reaction in the blood serum of three dogs in which the common bile duct was ligated and transected after they had been given viosterol in oil or drisdol by stomach tube. It was thus shown that vitamin D is not absorbed unless bile is present in the chyme.

*Excretion of Vitamin D in Bile of Two Dogs with Gallbladder Fistula after Intramuscular Injection of Vitamin D*  
 0 indicates no healing, (+) trace, ++ slight, +++ moderate, ++++ almost complete.

Dog	Operation date	Vitamin D injected			Bile collected	Vitamin D in dog bile shown by degrees of healing in x-rays of rachitic rats fed bile extracts		
		Preparation	Amount	Date		Amount	For 8 days	For 10 days
I	Jan. 25, 1937	Viosterol in oil	20	Feb. 4, 1937	Jan. 25, 1937-Feb. 1, 1937	cc.	0, 0	0, 0
					Feb. 1-4, 1937	19	0, 0	0, 0
					" 4-6, 1937	30	0, 0	0, 0
					" 6-8, 1937	19	0, 0	0, 0
					" 8-10, 1937	14	0, 0	0, 0
					" 10-12, 1937	13	0, 0	0, 0
					" 12-15, 1937	24	0, 0	0, 0
					" 15-19, 1937	120	0, (+)	(+), (+)
					" 19-23, 1937	136	0, 0	0, 0
					" 23-26, 1937	166	0, +	+, +
					" 26, 1937-Mar. 1, 1937	75	0, (+)	(+), +
					Mar. 1-5, 1937	27	0, 0	0, 0
					" 5-8, 1937	130	0, 0	0, 0
					" 8-13, 1937	97	0, 0	0, 0
K	Feb. 24, 1937	Drisdol	20	Feb. 27, 1937	" 20-27, 1937	28	0, 0	0, 0
					Feb. 24-26, 1937	72	0, 0	0, 0
					" 26-27, 1937	175	0	0
					" 27, 1937-Mar. 1, 1937	90	0, 0	0, 0
					Mar. 1-3, 1937	175	++, ++	++, ++
					" 3-5, 1937	63	++, ++	++, ++
					" 5-8, 1937	95	++, +	++, +
					" 8-10, 1937	112	0, 0	0, 0
					" 10-13, 1937	156	0, 0	0, 0
					" 13-15, 1937	145	+, +	+, +
					" 15-20, 1937	94	0, 0	0, 0
					" 20-23, 1937	125	0, +	0, +
						88		

Lack of absorption of vitamin D when crystalline vitamin D<sub>2</sub> in propylene glycol (drisdol) was given proves the importance of bile in the intestinal absorption of the vitamin molecule. The chemical nature of the solvent in these instances is irrelevant.

2. In six dogs with obstructed bile duct and in two dogs with gallbladder fistula, to which viosterol in oil or drisdol had been administered by intramuscular injection, vitamin D was excreted in bile in rather large amounts only after drisdol had been given. Excretion of vitamin D in bile when viosterol in oil was administered was surprisingly small in amount and possibly negligible. The chemical nature of the solvent, consequently, is of considerable importance in the excretion of vitamin D from liver tissue into bile.

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# METABOLISM AND MODE OF ACTION OF VITAMIN D

## V. INTESTINAL EXCRETION OF VITAMIN D

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The object of the experiments reported here was to trace the origin of vitamin D found present in feces after oral administration of vitamin D. The experimental conditions and the methods employed were those described in previous papers (1, 2), the only deviation being that for the detection of vitamin D in feces the dry weight of feces was used, instead of the fresh weight as for tissues, in calculating the dilution of the extract residue in oil.

When 25 cc.<sup>1</sup> of viosterol in oil, or of drisdol (crystalline vitamin D<sub>2</sub> in propylene glycol), were administered by stomach tube to healthy dogs weighing from 7 to 8 kilos (Dogs A and H, Table I), vitamin D was found present in the feces for 8 months after viosterol had been given and for at least 6 months after drisdol.

On the other hand, when intestinal absorption of vitamin D was made impossible by double ligation and transection of the common bile duct (2) and the same amount of viosterol in oil or drisdol was administered (Dogs B, D, and C, Table I), vitamin D was present in the feces for only from 10 to 16 days.

These results show that vitamin D given in oil or in propylene glycol is absorbed with the help of bile (2) and that whatever vitamin D is found in the feces 2 weeks after its administration has to be considered as excretion of previously absorbed amounts of vitamin D.

It has also been previously shown (2) that bile plays practically no rôle in the excretion of vitamin D given in oil; moreover, that excretion of vitamin D with bile, when vitamin D is given in

<sup>1</sup> 250,000 U.S.P. units of vitamin D.

TABLE I

*Length of Time Vitamin D Is Excreted in Feces of Dogs with and without Ligation and Transection of Common Bile Duct after Administration of Viosterol in Oil or Drisdol by Stomach Tube*

0 indicates no healing, (+) trace, + slight, ++ moderate, +++ almost complete, ++++ complete.

Dogs without ligation and transection of common bile duct										Dogs with ligation and transection of common bile duct									
Date of stool collection					Vitamin D shown by					Date of stool collection					Vitamin D shown by				
					For 8 days	For 10 days	Degree of healing* in x-rays of rachitic rats fed feces extracts	Determinations of blood serum of rachitic rats fed feces extracts for 10 days							For 8 days	For 10 days	Degree of healing* in x-rays of rachitic rats fed feces extracts	Determinations of blood serum of rachitic rats fed feces extracts for 10 days	
Dog								mg. per 100 cc.	Cal- cium	Dog								mg. per 100 cc.	Cal- cium
A					0	0		5.54	13.0	B					0	0		5.5	13.0
Mar. 31, 1936					0	0				Mar. 31, 1936					0	0			
Apr. 6, 1936					0	0				Apr. 6, 1936					0	0			
On Apr. 13, 1936, 25 cc. viosterol in oil given					+++	+++				On Apr. 15, 1936, operated					0	0			
Apr. 13-23, 1936					+++	+++				Apr. 18, 1936									
" 24, 1936-May 4, 1936					++	+++				On Apr. 20, 1936, 20 cc. viosterol in oil given					+++	+++			
May 4-14, 1936					+	++				Apr. 20-30, 1936					+++	+++			
" 24, 1936-June 4, 1936					0	0				" 30, 1936-May 10, 1936					0	0			
June 4-15, 1936					0	0				May 10-13, 1936					0	0			
" 15-25, 1936					++	++				On Sept. 3, 1936, operated									
" 25, 1936-July 6, 1936					0	(+)				Sept. 3-8, 1936					0	0			
Aug. 24, 1936-Sept. 2, 1936					0	(+)				On Sept. 8, 1936, 21 cc. viosterol in oil given					+++	+++			
Sept. 3-14, 1936					0	(+)				Sept. 8-12, 1936					+++	+++			

Nov. 5-15, 1936	0	0	4.71	11.5	Sept. 12-16, 1936	++	++	5.82	12.3
Dec. 1-12, 1936	+	++			" 16-21, 1936	++	++		
Jan. 10-20, 1937	0	0	5.05	12.5	" 21-24, 1936	++	++	5.05	13.4
Feb. 10-20, 1937	0	0	6.75	12.1	" 24-26, 1936	0	0		
Mar. 10-20, 1937	0	0	5.0	12.5	On Sept. 3, 1936, operated				
May 10-20, 1937	0	0			Sept. 15-29, 1936	0	0	7.3	11.5
Nov. 16-23, 1936	0	0			On Sept. 29, 1936, 25 cc. drisdol given				
On Nov. 23, 1936, 25 cc. drisdol given					Sept. 29, 1936-Oct. 1, 1936	++	++		
Nov. 23-28, 1936	+	++			Oct. 1-5, 1936	++	++	4.2	14.0
" 28, 1936-Dec. 4, 1936	+	++	7.1	11.5	" 10-15, 1936	+	++		
Dec. 4-12, 1936	0	0			" 15-25, 1936	0	0	3.3	15.0
" 12-17, 1936	0	0	6.27	11.5					
" 28, 1936-Jan. 6, 1937	(+)	(+)	4.7	12.8					
Jan. 6-16, 1937	(+)	+							
" 16-30, 1937	0	0	4.6	12.3					
" 30, 1937-Feb. 13, 1937	0	(+)							
Feb. 27, 1937-Mar. 13, 1937	0	0							
Apr. 1-12, 1937	0	0							
May 1-12, 1937	(+)	(+)							

\* Average values obtained from groups of two to three rats.

propylene glycol, is limited to the first 3 to 4 weeks after its administration. Consequently, the conclusion may be drawn that excretion after this length of time takes place exclusively through

TABLE II

*Excretion of Vitamin D in Feces of Three Dogs with Ligation and Transection of Common Bile Duct after Intramuscular Injection of Vitamin D*

0 indicates no healing, (+) trace, + slight, ++ moderate, +++ almost complete.

Dog	Date of operation	Vitamin D injected			Date of stool collection	Vitamin D shown by			
		Preparation	Amount	Date		Degree of healing* in x-rays of rachitic rats fed feces extracts		Determinations of blood serum of rachitic rats fed feces extracts for 10 days	
						For 8 days	For 10 days	Phosphorus	Calcium
	1936		cc.	1936	1936			mg. per 100 cc.	mg. per 100 cc.
B	Apr. 15	Viosterol in oil	20	May 13	Apr. 30-May 10	0	0	5.6	11.0
					May 10-13	0	0		
					" 13-25	++	+++	6.6	13.0
					" 25-June 4	0	0	4.7	9.6
					June 4-15	+	+	4.7	12.6
C	Sept. 3	Drisdol	25	Oct. 26	" 15-25	0	+	5.5	13.2
					Oct. 15-25	0	0		
					" 26-Nov. 2	(+)	(+)		
					Nov. 2- 9	+	+	5.3	12.3
					" 9-16	0	(+)	4.3	11.0
D	Nov. 19	Drisdol	25	Nov. 23	" 16-19	0	0	5.5	13.2
					" 13-19	0	0		
					" 19-23	0	0	5.3	12.3
					" 23-28	+	++	4.3	11.0
					" 28-Dec. 4	+	+		
					Dec. 4-9	0	(+)		

\* Average values obtained from groups of two to three rats.

the intestinal wall. Excretion of vitamin D after it has once been absorbed might start, however, as early as during the first 2 weeks. This is evident from experiments reported in Table II,

in which 20 to 25 cc. of viosterol in oil or of drisdol were given by intramuscular injection to three dogs in which the common bile duct had been ligated. In the first 5 days after its parenteral administration, vitamin D was excreted through the intestinal wall.

The rôle of the small intestines in excretion of vitamin D has been studied in experiments summarized in Table III. Seven dogs in which the common bile duct had been occluded or a gall-bladder fistula established were killed 1 to 21 days after parenteral

TABLE III

*Excretion of Vitamin D in Chyme and Feces of Five Dogs with Obstruction of Common Bile Duct and of Two Dogs with Gallbladder Fistula, after Intramuscular Injection of Vitamin D*

0 indicates no healing, (+) trace, + slight, ++ moderate.

Dog	Vitamin D injected		Time elapsed between injection of vitamin D and death of dog	Vitamin D shown by degree of healing* in x-rays of rachitic rats fed extracts of		
	Preparation	Amount		Chyme obtained from small intestine Upper one-third    Lower two-thirds	Feces obtained from large intestine only	
		cc.				
D	Viosterol in oil	20	1½ days	+		0
M†	" " "	25	5 "	(+)    0		(+)
I‡	" " "	20	7½ wks.	0    0		0
C	Drisdol	25	2 "	++		+
G	"	25	2 "	++		+
L†	"	25	5 days	++    +		+
K‡	"	20	3½ wks.	+    (+)		++

\* Average values obtained from groups of two to three rats.

† In these animals the pancreatic duct also was ligated.

‡ Bile fistula dogs.

administration of viosterol or drisdol. In two of the dogs with bile duct obstruction the pancreatic duct had also been ligated and transected. Immediately after death the intestines of these seven dogs were ligated at the pylorus, between the upper and middle third of the small intestine, at the ileocecal valve, and at the rectum. The contents of these different intestinal segments were then separately analyzed for the presence of vitamin D in the manner described (1, 2).

Vitamin D was excreted in the small intestines regardless of



whether it was dissolved in oil or in propylene glycol, as shown in Table III. It was excreted always in larger amounts in the upper third than in the lower two-thirds. Excretion through the wall of the large intestines cannot be discussed on the basis of these experiments. The presence of vitamin D in fecal material obtained from the large bowels obviously could be accounted for by its excretion through the duodenum and the jejunum.

#### SUMMARY

1. In three dogs with obstruction of the common bile duct, vitamin D administered by mouth as viosterol in oil or as crystalline vitamin D<sub>2</sub> in propylene glycol was present in the feces for from 10 to 16 days. In two animals not operated on, vitamin D remained demonstrable in the feces for from 6 to 8 months. These results show that after the first 2 weeks of oral administration vitamin D present in feces originates from a slow excretion of quantities that have first been absorbed.

2. In three dogs in which the common bile duct had been ligated and transected, excretion of parenterally administered vitamin D in feces started the first few days after injection.

3. Exclusion of bile from the intestinal tract in two dogs and obstruction of the pancreatic duct in two other dogs proved, beyond doubt, that vitamin D, administered by intramuscular injection in the form of viosterol in oil or of drisdol, is excreted through the intestinal wall. By analysis of the content of the different intestinal segments separately for the presence of vitamin D, after the animals had been killed, it was found that vitamin D was excreted in rather large amounts through the wall of the upper third of the small intestines. The rôle played by the large intestines in the excretion of vitamin D cannot be discussed on the basis of the experiments reported.

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## DETERMINATION OF SULFANILAMIDE IN BLOOD AND URINE\*

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The wide-spread use of sulfanilamide (*p*-aminobenzenesulfonamide) in the treatment of various bacterial infections in human beings, as well as its marked therapeutic effect in certain infections in animals, makes it desirable to have an accurate method for determining the substance in blood, urine, and various body fluids. The present method is based on the diazotization of the *p*-aminobenzenesulfonamide with nitrous acid, and the coupling of the resulting diazo compound in acid solution with dimethyl- $\alpha$ -naphthylamine to produce a purplish red azo dye which can be easily estimated by colorimetric comparison.

This reaction depends on the presence of an amino group substituted in the benzene ring and probably can also be used to estimate any derivative of sulfanilamide in which the amino group is free or can be freed by hydrolysis. It has been used successfully with certain derivatives of sulfanilamide where various substitutions are made in the sulfonamide group and with readily hydrolyzable derivatives with substitution in the amino group. Filtrates from blood of normal man or of various animals or normal urines do not appear to contain any substance in sufficient amounts to give the reaction. This color reaction is exceedingly delicate, being detectable in a solution of sulfanilamide of 1 part in 20 million parts of water.

In the mouse, rat, cat, rabbit, monkey, and man, but not in the dog, sulfanilamide is partly conjugated with acetic acid and excreted as the acetyl derivative (3). This compound can be

\* Preliminary accounts of this method have been published (1, 2).

determined by the above method in urine and blood filtrates after hydrolysis.

*Reagents*<sup>1</sup>—

1. A solution of *p*-toluenesulfonic acid containing 20 gm. dissolved in water and diluted to 100 cc.

2. A 0.1 per cent solution of sodium nitrite.

3. A solution of dimethyl- $\alpha$ -naphthylamine containing 1 cc. in 250 cc. of 95 per cent ethyl alcohol. This solution should be kept in a dark colored bottle.

4. A solution of saponin containing 0.5 gm. per liter.

5. 1 N hydrochloric acid.

6. 2 N sodium hydroxide.

7. A 0.1 per cent solution of phenolphthalein in ethyl alcohol.

8. A stock solution of sulfanilamide in water containing 200 mg. per liter. A weighed quantity of crystalline sulfanilamide is dissolved in hot water and diluted to appropriate volume. This solution appears to keep unchanged for several months in the ice box. From the stock standard solution, standards for use are prepared. The most convenient ones are 1, 0.5, and 0.2 mg. per cent. To prepare these in water 5, 2.5, and 1 cc. of the stock solution are diluted to 100 cc. To prepare these standard solutions containing toluenesulfonic acid for use in the blood method,

<sup>1</sup> The *p*-toluenesulfonic acid and dimethyl- $\alpha$ -naphthylamine can be obtained from the Eastman Kodak Company, Rochester, New York. The dimethyl- $\alpha$ -naphthylamine should be tested for suitability for use in the method by running a blank with distilled water and by determining its speed of coupling in a dilute solution of sulfanilamide. In the blank only a very slight yellow color should develop in 10 minutes and a yellowish brown in 2 hours with no red or purple tint. When a 1 mg. per cent solution of sulfanilamide is diazotized and treated with the solution of dimethyl- $\alpha$ -naphthylamine, maximum color development should be attained in less than 10 minutes. We have had samples (presumably containing small amounts of unmethylated  $\alpha$ -naphthylamine) which gave so much color in the blank as to be unsatisfactory for use in the method. We have recently had several pure samples which reacted extremely slowly with diazotized sulfanilamide. Apparently a small amount of an oxidation product of dimethyl- $\alpha$ -naphthylamine is necessary as a catalyst for rapid color development in dilute solutions. Samples which give very slow color development can be made satisfactory for use in the method by heating to 265° for 10 minutes while a moderate stream of air is being passed into the liquid through a capillary tube. We have found it convenient to heat 5 or 10 cc. portions at a time, using a large test-tube in an oil bath.

5, 2.5, and 1 cc. of the stock solution plus 18 cc. of the solution of toluenesulfonic acid are diluted to 100 cc. These standard solutions can be used for at least 1 week.

*Procedure for Blood*—2 cc. of oxalated blood are measured into a flask and diluted with 14 cc. of the saponin solution. After laking is complete (1 or 2 minutes), 4 cc. of the solution of *p*-toluene-sulfonic acid are added with shaking.<sup>2</sup> The mixture is filtered after 5 minutes, and 10 cc. of the filtrate are measured into a small flask or test-tube. 1 cc. of the solution of sodium nitrite is added, and the solution shaken. After 3 minutes standing, 5 cc. of the solution of dimethyl- $\alpha$ -naphthylamine are added from a burette. 10 cc. of a standard solution of sulfanilamide containing toluene-sulfonic acid are treated like the blood filtrate, and the color compared with the unknown. The comparison can be made at any time from 10 to 60 minutes after addition of the naphthylamine. A 1 mg. per cent standard is satisfactory for bloods containing from 5 to 20 mg. per cent of sulfanilamide; for lower values a weaker standard is used, while blood containing more than 20 mg. per cent is diluted to a greater degree before precipitating the proteins.

If it is desired to determine the conjugated sulfanilamide (probably *p*-acetylaminobenzenesulfonamide)<sup>3</sup> as well as the free sulfanilamide in blood, a 1:20 instead of a 1:10 blood filtrate is prepared. 2 cc. of blood are diluted with 30 cc. of saponin solution, and precipitated with 8 cc. of the solution of toluenesulfonic acid. The free sulfanilamide is determined in this filtrate as described above. To estimate the total sulfanilamide, 10 cc. of the filtrate are placed in a test-tube (15  $\times$  150 mm.) graduated at

<sup>2</sup> If saponin is not available, the blood can be diluted with distilled water and allowed to stand for 15 minutes before the toluenesulfonic acid is added. If laking is not complete, low results are obtained. A smaller amount than 10 cc. of filtrate (allowing the use of less blood) can be used by appropriate reduction of the amounts of the nitrite and dimethyl- $\alpha$ -naphthylamine reagents.

<sup>3</sup> Acetylsulfanilamide has been isolated from the urine of the rabbit and man after administration of sulfanilamide (3). The amount of the acetyl derivative excreted appears to depend upon the amount of conjugated compound present in the blood. It seems almost certain that the conjugated compound in blood is acetylsulfanilamide and we have used pure acetylsulfanilamide in checking our analytical procedure.

10 cc. and heated in a boiling water bath for 90 minutes. The solution is then allowed to cool and diluted to 10 cc. to replace the water lost by evaporation. The procedure is now the same as that used in determining the free sulfanilamide. The difference between the values obtained before and after hydrolysis gives the amount of acetylsulfanilamide (calculated as sulfanilamide).<sup>4</sup>

*Procedure for Urine*—Urine is ordinarily diluted so that the diluted solution contains from 0.5 to 2.0 mg. per cent of sulfanilamide. 10 cc. of this diluted urine are treated with 1 cc. of the solution of toluenesulfonic acid, and then with 1 cc. of the nitrite solution. After 3 minutes standing, 5 cc. of the solution of dimethyl- $\alpha$ -naphthylamine are added. A standard solution of sulfanilamide in water (usually 1 mg. per cent) is treated in the same way. After 10 minutes, the solutions are compared in the colorimeter. Ordinarily dilutions of 1:50, 1:100, or 1:200 are satisfactory. Urine more concentrated than 1:25 should not be used, as substances of the urine may interfere with color development. With only small amounts of sulfanilamide present, a weaker standard should be used.

To determine the acetylsulfanilamide in urine, 1 cc. of urine is placed in a test-tube, treated with 2 cc. of 1 N hydrochloric acid, covered to prevent evaporation, and heated in a boiling water bath for 30 minutes. The solution is allowed to cool and neutralized with 2 N sodium hydroxide after the addition of 1 drop of phenolphthalein solution. The mixture is now diluted to appropriate volume (1:50 to 1:200) and 10 cc. of the diluted solution taken for determination of total sulfanilamide by the method described for urine. The difference between the total and free gives the acetylsulfanilamide (calculated as sulfanilamide). If only small amounts of sulfanilamide are present in urine, so that a dilution of 1:50 is too weak to compare accurately with a standard, it is more accurate to treat the urine by the blood method (1:20 dilution) for determining both free and conjugated sulfanilamide.

*Colorimetric Comparison*—It is essential to use all-glass cups in the colorimeter, as the ordinary glass cup with detachable glass bottom may give inconsistent and erroneous results. This is

<sup>4</sup> Since acetylsulfanilamide yields 80.3 per cent of sulfanilamide, one can obtain figures for the acetyl compound as such by dividing by 0.803.

apparently due to a trace of metal dissolved from the metal cup shield. The 1 mg. per cent standard is conveniently set at 10 mm. and the weaker ones at 15 or 20 mm.<sup>5</sup> The color development is strictly proportional for the range of concentrations between one-half and twice those of the standard. This is shown in Fig. 1.

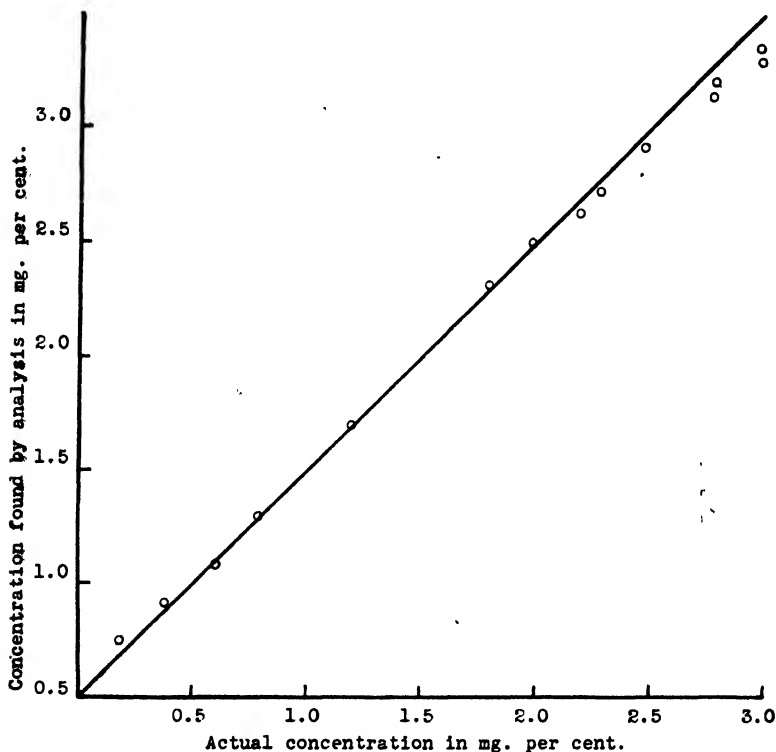


FIG. 1. The values found for different concentrations of sulfanilamide read against a 1 mg. per cent standard.

<sup>5</sup> The use of a green filter (Wratten light filter No. 61 N or Jena glass Filter VG, No. 2) facilitates the comparison, especially with weak colors. We have found that the proportionality of the color with respect to concentration does not have as great a range as when no filter is used. With a 1 mg. per cent standard, solutions containing 0.5 to 1.5 mg. per cent read correctly but a 2 mg. per cent solution gives results about 4 per cent low. All results reported in this paper were obtained without the use of a filter.

*Variations in Amounts of Reagents*—The amount of toluenesulfonic acid added can be varied considerably without affecting the accuracy of the determination. A 4 per cent solution of toluenesulfonic acid (20 cc. of 20 per cent solution diluted to 100 cc.) gives a pH of about 0.9, while a 2 per cent solution gives a pH of about 1.1. A 3.6 per cent solution (18 cc. of 20 per cent diluted to 100 cc.) gives a pH of about 1.0. A pH of about 1.0 appears best for carrying out the diazotization and coupling with the naphthylamine.<sup>6</sup> Blood filtrates (prepared as above) average pH 1.0, so that the standard solutions are adjusted to this hydrogen ion concentration. The addition of toluenesulfonic acid to the diluted urine gives a pH of about 1.0. No difference is observed in solutions treated with amounts varying from 0.7 to 1.5 cc. of nitrite solution to 10 cc., 1.0 cc. being added to the standard. About 1 minute is necessary to complete the diazotization, but since no difference in the results was observed with an 8 minute wait, we have allowed 3 minutes for completion of the reaction. For proportionality of the color developed in solutions one-half to twice as strong as the standard, 5 cc. of the dimethyl- $\alpha$ -naphthylamine solution are necessary. Moreover, with less dimethyl- $\alpha$ -naphthylamine the presence of small amounts of salts is liable to retard color development and lead to erroneous results. Since the azo dye formed in the reaction is difficultly soluble in water, a certain amount of alcohol is necessary to prevent turbidity. The alcohol is added with the dimethyl- $\alpha$ -naphthylamine.

*Protein Precipitants for Blood*—The original procedure described for blood (1), with ethyl alcohol as the protein precipitant, gives accurate results, but does not allow a determination of the conjugated compound. Moreover, the alcohol procedure gives weaker colors and is more laborious than the present method. Various other protein precipitants were tried. Trichloroacetic acid gives accurate results for the free sulfanilamide, but due to decomposition on heating does not completely hydrolyze the conjugated compound. Benzenesulfonic acid was found to be satisfactory, but since this compound is hygroscopic and comparatively expensive, the *p*-toluenesulfonic acid was substituted. The

<sup>6</sup> When hydrochloric acid is used as in our original method, the pH should be about 2.0. The use of toluenesulfonic acid is much more satisfactory than hydrochloric acid.

~~Folin~~ tungstate, Somogyi copper and iron, and metaphosphoric acid filtrates were found to be unsatisfactory.

**Recovery from Blood**—Tables I and II illustrate the recovery of sulfanilamide and *p*-acetylaminobenzenesulfonamide added to blood and precipitated with toluenesulfonic acid in 1:10 and 1:20 dilution. The recovery of sulfanilamide is quantitative either in a 1:10 or 1:20 dilution, but the recovery of acetylsulfanilamide is

TABLE I

*Recovery of Sulfanilamide and Acetylsulfanilamide Added to Blood.  
Precipitation in 1:10 Dilution*

In all tables, "free" refers to unconjugated sulfanilamide and total to sulfanilamide plus the acetylsulfanilamide (calculated as sulfanilamide).

Species	Added		Found	
	Free	Total	Free	Total
	mg. per cent	mg. per cent	mg. per cent	mg. per cent
Human	6.7	9.3	6.8	7.9
"	6.7	14.7	6.7	14.1
"	6.7	20.0	6.7	17.0
"	10.0	14.0	10.1	12.2
"	10.0	18.0	9.8	15.7
Rabbit	0.0	8.0	0.0	7.3
"	5.0	17.0	5.0	16.0
"	6.7	14.7	6.7	11.8
"	8.3	15.0	8.4	13.5
Dog	1.0		1.2	
"	3.0		3.3	
"	4.0		4.1	
"	6.6		6.4	
"	10.0		10.0	
"	13.3		13.3	
"	15.0		14.7	
"	20.0		19.6	

much more complete in the 1:20 than in the 1:10 dilution. The results are, however, slightly low for the acetyl derivative. Table III shows a few determinations of free and total sulfanilamide in blood taken from patients receiving the drug. It is evident that the 1:20 dilution gives higher values than the 1:10 for total sulfanilamide.

**Recovery from Urine**—Table IV shows the recovery of sulfanilamide and acetylsulfanilamide added to urine.



*Hydrolysis of Acetylsulfanilamide*—1 cc. of a 100 or 200 mg. per cent solution of *p*-acetylaminobenzenesulfonamide, treated with 2 cc. of 1 N hydrochloric acid and heated, is completely hydrolyzed

TABLE II  
*Recovery of Sulfanilamide and Acetylsulfanilamide Added to Blood.  
Precipitation in 1:20 Dilution*

Species	Added		Found	
	Free	Total	Free	Total
	mg. per cent	mg. per cent	mg. per cent	mg. per cent
Human	5.0	9.0	5.2	8.6
"	10.0	14.0	9.9	13.5
"	10.0	18.0	10.1	17.9
"	10.0	18.0	9.8	17.1
"	15.0	19.0	15.0	18.5
"	15.0	23.0	14.9	22.0
"	20.0	24.0	19.6	23.5
Rabbit	6.7	10.7	6.8	10.3
"	8.3	15.0	8.6	14.6
"	8.3	15.0	8.3	14.3
"	10.0	18.0	9.9	17.7
"	10.0	18.0	9.8	17.7

TABLE III  
*Sulfanilamide and Acetylsulfanilamide in Blood of Patients after  
Administration of Drug*

Subject No.	1:10 dilution		1:20 dilution Total
	Free	Total	
	mg. per cent	mg. per cent	mg. per cent
1	3.7	5.6	5.6
2	7.5	12.1	12.5
3	8.8	13.2	14.4
4	9.1	9.9	10.3
5	14.3	17.0	17.9
6	15.6	17.9	19.8

in 20 to 25 minutes and can be heated for 2 hours or more with no further change. Solutions of sulfanilamide were heated with 1 N hydrochloric acid for 2 hours with no change in concentration detectable by the colorimetric method. We have carried out

several hundred analyses of acetylsulfanilamide in urine by heating 1 cc. of urine with 1 cc. of 1 N hydrochloric acid for 30 minutes and controlling the hydrolysis by heating for 60 minutes or by using 2 cc. of 1 N hydrochloric acid. In only a few instances (in the case of very alkaline urines) did the heating with 1 cc. of 1 N acid for 30 minutes fail to effect complete hydrolysis. We now use as a routine 2 cc. of 1 N acid with 30 minutes heating. The sodium chloride formed from the neutralization of the hydrochloric

TABLE IV  
*Recovery of Sulfanilamide and Acetylsulfanilamide from Urine*

Species	Dilution	Added *		Found	
		Free	Total	Free	Total
		<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>
Human	1:20	10.0	18.0	10.4	18.4
"	1:25	10.0	26.0	10.3	26.3
"	1:25	20.0	28.0	19.6	28.8
"	1:25	20.0	60.0	20.0	59.5
"	1:50	20.0	60.0	19.8	59.5
"	1:50	20.0	100.0	20.1	96.0
"	1:50	100.0		100.0	
"	1:100	20.0	100.0	20.1	101.0
"	1:100	40.0	200.0	40.5	192.0
"	1:100	100.0		101.0	
"	1:100	200.0		200.0	
Rabbit	1:25	25.0		24.0	
"	1:50	0.0	80.0	0.0	80.5
"	1:100	100.0		100.0	
Dog	1:25	20.0		19.1	
"	1:100	20.0	100.0		101.0
"	1:100	40.0		40.1	

acid, if in sufficient concentration, interferes with the determination and leads to low results. A dilution after hydrolysis to 50 cc. or more is satisfactory, but if dilution is made to only 25 cc., slightly low results may be encountered. This can be avoided by using the blood procedure with toluenesulfonic acid with a dilution of 1:20.

In the blood procedure, the toluenesulfonic acid is present in sufficient concentration in the filtrate to effect hydrolysis of the acetyl compound. A 1 mg. per cent solution of sulfanilamide

containing 3.6 per cent toluenesulfonic acid can be heated in the water bath for 60, 90, or 120 minutes with no change in the amount of sulfanilamide present. A solution of acetylsulfanilamide (1 mg. per cent) with or without the addition of sulfanilamide in 3.6 per cent toluenesulfonic acid is hydrolyzed completely by 60 minutes heating. Blood filtrates have been heated for the usual 90 minutes and also for 120 minutes with agreement of the values obtained for total sulfanilamide.

*Body Fluids Other than Blood and Urine*—Sulfanilamide and its acetyl derivative can be estimated in other body fluids by the same procedure as for blood. Plasma, serum, cerebrospinal fluid, chest fluid, and ascitic fluid, as well as pancreatic juice and saliva, have been used. A method based on our original alcohol procedure for blood (1) has been developed for the estimation of sulfanilamide in tissues (4).

#### DISCUSSION

The method described for determining sulfanilamide and its acetyl derivative in blood and urine appears to be satisfactory and sufficiently accurate for most purposes for which it may be used. The coupling in acid solution prevents many substances present in the body fluids from interfering. A number of other substances were tried for coupling with the diazo compound but none gave the sensitivity of dimethyl- $\alpha$ -naphthylamine. Fuller (5) has described a method for determining sulfanilamide in urine by diazotizing and coupling with  $\beta$ -naphthol in alkaline solution, and in blood by precipitation with trichloroacetic acid, adding sodium nitrite to the filtrate, and coupling in alkaline solution with thymol. No details of the method are given, but from our experience with coupling of the diazo compound in alkaline solution, we judge it to be less satisfactory than the present method.

As stated above, our method can be used for determining derivatives of sulfanilamide in which the amino group is free, or in which this group can be freed by hydrolysis. A standard of the particular compound should be used and control recoveries from blood and urine performed before trusting the method for a new compound. The amount of alcohol used may have to be increased in certain cases to prevent turbidity. It is probable that certain other compounds containing a free amino group in the benzene ring can also be estimated by this procedure.

## SUMMARY

A method is described for the determination of sulfanilamide in urine, blood, and other body fluids. This method is based on diazotization of the sulfanilamide and the subsequent coupling of the diazo compound with dimethyl- $\alpha$ -naphthylamine to form a purplish red dye which can be estimated by colorimetric comparison. Acetylsulfanilamide is hydrolyzed to sulfanilamide, which is then determined. The method appears applicable to certain derivatives of sulfanilamide.

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# THE UTILIZATION OF FRACTIONS OF THE NITROGEN PARTITION OF THE BLOOD BY THE ACTIVE MAMMARY GLAND\*

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The experiments reported by Cary (1), Blackwood (2), and Lintzel (3) show that amino acids are removed from the blood during its passage through the lactating mammary gland. The fact that the inactive mammary gland does not absorb amino acids in quantities which may be observed as differences in comparisons of arterial and mammary blood analyses has been accepted as proof that its absorption is associated with milk secretion and that amino nitrogen is the precursor of milk nitrogen.

During investigations on the nutrition of the mammary gland being carried out in this laboratory, there has been occasion to study some parts of the nitrogen metabolism of the mammary gland. The technique used throughout these experiments was that previously described (Graham *et al.* (4, 5)), in which samples of blood were taken simultaneously from the mammary vein and the carotid artery of the goat. The oxalated blood samples so taken were subjected to various types of analyses in an attempt to find changes in their composition which might result from passage through the actively secreting mammary gland.

The result of the analyses of four typical pairs of blood samples for amino N (Danielson (6)) and urea (Van Slyke (7)) are shown in Table I.

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## 276 Nitrogen Utilization by Mammary Gland

These results which are typical of several additional experiments demonstrated clearly that the loss of urea nitrogen from the gland to the venous blood often exceeds the absorption of amino N from the same blood. This condition appears to exist generally in early lactation except during the early hours of the period between milkings, when urea is in positive balance. The production of urea falls off considerably during the latter stages of lactation. At this time considerably more amino N is absorbed than urea N is excreted.

Experiments in which the blood volume flow through the mammary gland was measured indicate that the ratio of blood volume flow to milk secretion is between 150:1 and 250:1. These findings are slightly higher than those published by Jung (8), as determined

TABLE I

*Showing Level of Urea and Amino Nitrogen in Arterial and Mammary Venous Blood during Early Lactation*

The values are given in mg. per cent.

Amino N			Urea N		
Arterial	Venous	Difference (arterial minus venous)	Arterial	Venous	Difference (arterial minus venous)
9.60	8.64	0.96	28.08	29.18	-1.10
9.55	8.86	0.69	29.65	30.94	-1.29
9.80	8.70	1.10	25.92	27.49	-1.57
7.74	7.38	0.36	32.70	33.69	-0.99

by direct measurement, and considerably lower than those calculated by Lintzel (3) and Graham *et al.* (9) from arterial-venous constituent analyses. These calculations may be considered valid only when the precursors of any milk constituent are accurately known. Lintzel used a blood sugar to lactose and amino N to milk N conversion for his calculations. Graham *et al.* used blood sugar to lactose and blood fat to milk fat in their estimations. The basis for these calculations has since been proved to be erroneous by the demonstrations of additional precursors for the production of milk solids. In experiments previously published, Graham (10) has shown that the arterial-venous values for amino N could not supply the total nitrogen necessary for the formation of milk when the blood volume flow through the mammary gland

was determined by the thermostromuhr method. It therefore appeared from the evidence cited that amino N could not be the sole precursor of milk N and that, as previously suggested, it may not enter into the reactions for the formation of milk N to any large extent.

In the course of other studies with goats during early lactation, the total and amino nitrogen contents of the arterial and mammary venous blood were determined. The results are shown as

TABLE II

*Showing Arterial-Mammary Differences Found in Total Nitrogen Content of Blood Taken from Lactating Goats*

The values are given in mg. per cent.

Goat No.	Time since milked last			
	1-2 hrs.	4-5 hrs.	7-9 hrs.	14-15 hrs.
403	-57	-19	0	20
348	-230	43	20	36
30	-79	-20	24	-14

TABLE III

*Showing Arterial-Mammary Differences Found in Amino Nitrogen Content of Blood Taken from Lactating Goats*

The values are given in mg. per cent.

Goat No.	Time since last milking			
	1-2 hrs.	4-5 hrs.	7-9 hrs.	14-15 hrs.
30	0.95	0.36	0.20	0.73
348	1.37	0.96	0.69	0.27
403	0.69	0.32	0.96	0.27

the arterial-mammary differences in Tables II and III, respectively. Total nitrogen was determined by the Kjeldahl procedure and amino nitrogen by the method of Danielson. These data were not corrected for possible water loss from the blood. These data add further confirmation to the previous observations that amino N is removed from the arterial blood by the mammary gland during lactation (Table III), but suggest that our knowledge of the nitrogen metabolism of the active mammary gland is in need of further investigation.



## 278 Nitrogen Utilization by Mammary Gland

In a continuation of these studies, arterial-mammary blood comparisons were made with five lactating goats. The pairs of blood samples were taken at three intervals after milking, 1 to 2 hours, 4 to 5 hours, and 6 to 8 hours, respectively. All samples were taken between 8 a.m. and 6 p.m., but not on the same day. The animals were in a declining phase of the lactation cycle when the experiments were performed.

In most cases the blood samples were taken in vessels coated with potassium oxalate. A limited number, however, were taken for serum determinations without anticoagulants. The oxalated blood was chilled in ice water, while the serum was kept at room temperature. All precipitations were completed within 1½ hours after the blood was taken, except serum, which separated in 4 to 5 hours. In no case were samples allowed to stand overnight. The following methods of analyses were used: total N, semimicro-Kjeldahl; albumin and globulin, Howe (11); urea, Van Slyke (7); amino N, Danielson (6); non-protein N, Folin and Wu (12); amide N, Bliss (13); fibrin, Howe (11); and hemoglobin (14). The actual determination of nitrogen in the micromethods for non-protein N and amide N was made by the manometric method of Van Slyke instead of the colorimetric nesslerization procedure. The results recorded were the averages of duplicate determinations agreeing within 2 per cent.

A correction for water loss, on the basis of hemoglobin determinations (averaging less than 1 per cent), was made.

The results of the analyses presented as the arterial-mammary vein differences confirm the previous analyses in that amino N is consistently removed from the arterial blood by the mammary gland. The negative values found in the previous experiments for the total nitrogen determinations on blood are similar in both blood and plasma data to those shown in Table II. Since these data have been corrected for the small water loss, the negative balance in the nitrogen metabolism of the gland at this time is demonstrated. Later in the period between milkings a positive balance is attained as shown in Tables II and III.

The differences in the total nitrogen content between the arterial and mammary vein blood samples cannot be accounted for in the non-protein nitrogen of the blood. Of fifteen determinations of this fraction, twelve indicated that non-protein nitrogen as a

TABLE IV  
*Showing Arterial-Mammary Differences for Nitrogen Partition of Blood of Lactating Goats (Corrected for Water Loss)*

Experiment No.	Blood differences, mg. per cent N				Plasma differences, mg. per cent N					Remarks
	Total	Non-protein	Amino	Urea	Total	Fibrin globulin	Non-protein N + albumin	Non-protein	Amide	Fibrin
1	4	3.55	1.25	-1.00	5	-29	34	3.44	10	
2	-92	2.30	0.38	0.48	-44	14	-57	2.46	5	
3	-12	-2.47	0.55	-0.49				-2.70	-14	-10
4	-20	2.28	0.85	-0.23	-17*	-45*	-28*	-3.77	-63	-2
5	-34	0.16			-10	145	-156	5.01	-14	
6	-5	-2.55	1.12	-0.56						
7	51	7.46		-0.97	52	44	8			-23
8		2.48			5	58	-252			
9	82	7.41	0.56	-0.34	103	120	-12	-2.96	20	
10	-54	7.89	0.72	-0.10	189*	253*	-63*			
11	9				-23	103	-126	8		
12	9	9.18		1.02	53	-16	78			-34
13					30	91	-61	0		
14					20	20	0			
15					0	-34	34			
16					61	91	-30			
17	2.48									
18	3.98	2.52	2.46		90	101	-11	2.15	-2	6
19	5.1	-21.15	1.38	0.46	6					
20	6	6.21	0.33							
21										
22										
23										
24										
Mean	-0.38	1.95	0.96	-0.49	37	62	-40	0.52	-6	-13

\* Determinations on serum.

## 280 Nitrogen Utilization by Mammary Gland

unit was being taken up by the gland. The amounts taken up indicate that fractions other than may be accounted for by the urea or amino N are entering into the picture of the metabolism of the active mammary gland. Eleven comparisons of the urea contents of these bloods showed nine cases in which urea was being produced.

The most remarkable feature shown in Table IV of comparisons lies in the determination of the albumin and globulin of the plasma. The arterial-venous differences for nineteen pairs of plasma or serum are shown. In sixteen cases these show that globulin is being taken up by the mammary gland in rather large amounts. At the same time the albumin fraction of the venous blood shows increases as indicated by the negative values of the differences. Unfortunately, the method used to determine globulin is indirect; namely, the difference between total nitrogen and albumin plus non-protein nitrogen. The globulin fraction thus determined includes the globulins and fibrin. Arterial-mammary differences are shown for a limited number of fibrin and amide nitrogen determinations; however, little significance can be attached to these because of the limited number of data. It is, however, interesting to note that the negative balance of the gland in the early part of the period between milkings might be partially accounted for by the amide N. The production of a compound excreted into the mammary blood and precipitated as fibrin is indicated in the results. It is possible that had this fraction been determined through the experiments the negative values shown for globulin in the three cases might have been modified as shown in Experiment 13 (Table IV).

### DISCUSSION

The results of these experiments show that amino N, globulin, and unknown fractions of the non-protein N of the blood are taken up by the active mammary gland. A fraction of the protein nitrogen determined as albumin appears to be returned to the blood. These observations confirm the findings of Graham (10), in which it was shown that amino nitrogen could not form all of the nitrogen of the milk. The data indicate that milk nitrogen is derived chiefly from the globulin of the blood. The uptake of

this substance as well as the non-protein N, including amino acids, appears to be continuous throughout the hours between milkings studied.

The results of total nitrogen determinations on the blood and plasma (Tables II and IV) show that the gland goes through a cycle of nitrogen metabolism during the interval between one milking and the next. The results of nearly all experiments reported in the literature dealing with the time of milk secretion indicate that milk production is most rapid in the early hours after the gland has been emptied. Following this the rate of secretion diminishes owing to the pressure of the stored secretion upon the secreting tissue. These experiments, however, show that during the period of most active milk secretion the actual balance of nitrogen metabolism of the mammary gland aside from the production of milk is negative. This balance gradually changes to a positive one in a few hours. Since the time of milk secretion is well established, these results may mean that the milk nitrogen secreted shortly after milking is in a large part derived from nitrogenous compounds which have been stored within the gland during the later hours of the previous interval.

It might be mentioned that following the removal of milk there would be a considerable reduction of udder pressure. As a consequence there may be an increased inflow of blood plasma into the intercellular spaces of the udder. Several hours might elapse before equilibrium between the inflow of milk precursors and the return of the products of mammary cell synthesis and metabolism was reestablished. The negative nitrogen balance observed, due to differences in the rate of inflow and outflow of blood, might be more apparent than real. This phase of the problem requires further study.

The determinations of albumin and globulin show that globulin is taken up from the incoming blood and is probably used for the production of milk nitrogen. The complete set of analyses indicates, as a whole, that a large part of the nitrogen so taken up is returned to the venous blood in the albumin, non-protein nitrogen, amide, or fibrin fractions. If this is true, it would appear that the milk nitrogen is probably not built up piece by piece from amino acids but that part of the molecule of globulin is used for

## 282 Nitrogen Utilization by Mammary Gland

the synthesis together with fractions of the non-protein nitrogen, including amino acids. The remaining portions not needed for the synthesis are then returned to the blood.

A conception of the formation of the nitrogenous fractions of milk as outlined would explain certain relationships which exist between the constituents of milk on a more justifiable basis than could be used if the protein nitrogen of milk is assumed to be derived from the amino acids alone. In this the osmotic changes due to the formation of casein from amino nitrogen as suggested by Blackwood and Stirling (15) must be slightly modified, as the maximum change that could take place would be that from globulin, a lyophilic compound, to casein, which is lyophobic. In this case the drop in osmotic pressure so useful in explaining the rapid secretion of milk, must be due largely to the formation of lactose from glucose and lactic acid. The rôle of the utilization of amino nitrogen will probably be of less importance than previously anticipated.

There can be little doubt that the secreting cells of the mammary gland are permeable to globulin, since milk and particularly colostrum contain globulin which is serologically identical with that found in the blood. The transition from a colostrum of high globulin content could, under these conditions, be explained without assuming a change in the permeability of the secreting cell membrane. Such a change is most unlikely, particularly in view of the fact that the more rapid secretion of milk, as compared with the secretion of colostrum, would have to be associated with a membrane of a less permeable nature.

There can be little doubt that the casein and lactose of the milk depend upon enzyme systems for their formation, though these have not been thoroughly demonstrated at the present time. Bergman and Turner (16) have shown that the amount of lactose secreted is in some manner dependent upon the amount of lactogenic hormone injected into their experimental animals. Consequently, it would seem more reasonable to suppose that milk secretion is dependent upon the hormones activating or forming the enzyme systems which utilize the precursors from the blood rather than attempt to explain a radical change in the permeability of the cells of the mammary gland at parturition.

## SUMMARY

The arterial-mammary venous differences in the nitrogen partition of blood samples simultaneously taken from goats actively secreting milk showed that the mammary gland was utilizing globulin, amino acids, and undetermined fractions of the non-protein nitrogen partition of the blood. Large amounts of nitrogen were returned to the albumin fraction of the venous plasma.

The mammary gland is in negative nitrogen equilibrium for some time after the animals are milked. This changes to a positive balance which may continue to exist until the next milking. The results indicate that nitrogen may be stored in the mammary gland for utilization in the future production of milk.

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## ON THE FORM OF COPPER IN BLOOD PLASMA\*

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(Received for publication, October 18, 1937)

Although copper has been the subject of numerous researches which have demonstrated that it is essential for normal hemoglobin formation, there have been very few attempts to discover the type of compound in which the element occurs in the case of red blooded animals.

Warburg and Krebs (1) approached the problem in experiments on what was referred to as loosely bound copper in blood serum. Using the Warburg apparatus, they found that blood serum plus 1 volume of 0.2 N HCl would catalyze the oxidation of cysteine just as effectively as the ash of the serum. These workers calculated the copper content of the serum on the basis of its catalytic activity. Tompsett (2) reported that trichloroacetic acid filtrates contained all of the copper of the original blood. Eisler, Rosdahl, and Theorell (3) found the rates of migration of copper ions to be the same as that of serum albumin molecules in cataphoresis experiments with horse serum. From this they concluded that the copper and the albumin were combined into a complex. They supported their contention with the observation that when the protein was precipitated below its isoelectric point the copper went into the filtrate, and when the protein was precipitated above the isoelectric point, the filtrate was free from copper.

There have been a number of studies on "loosely bound" or "easily split off" iron in blood, chiefly by Barkan and coworkers. Moore *et al.* (4, 5) have recently published an extensive investiga-

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† On leave of absence from the Kentucky Agricultural Experiment Station, Lexington, Kentucky, and published with the permission of the Director.



tion of this subject and include a complete bibliography. Easily split off iron has been determined by a number of methods. One of these has been to add 1 volume of 0.2 N HCl to the blood plasma and dialyze. The easily split off iron was then determined in the dialysate.

Abderhalden and Möller (6) reported that virtually no copper can be removed from serum by dialysis and concluded that the copper was in organic form in the serum. Schultze, Elvehjem, and Hart (7) showed that in nutritional anemia the copper content of blood was markedly lowered. The present work was undertaken in an attempt to determine the form of copper in the blood, and is concerned with the dialysis of plasma copper under various conditions.

#### EXPERIMENTAL

Whole blood was collected from one cow over a period of several months.<sup>1</sup> 2 mg. of copper-free potassium oxalate per cc. of blood were used to prevent clotting. The plasma was obtained by centrifuging. The total copper content was determined on a 10 cc. portion of the plasma. Aliquots of the plasma with and without acid were then placed in cellophane sacks and dialyzed against 20 volumes of redistilled water for 24 hours at 2-4°. The dialysis sacks were made from sections of cellophane tubing (du Pont transparent seamless tubing, 1½ inch) which were attached to glass necks, and supported by wooden collars in order to prevent copper contamination. Since the total copper content of the plasma in the sacks was only 10 to 15 micrograms, it was necessary to exercise extreme precautions throughout. The dialyzed and non-dialyzed copper is expressed in terms of percentage of the total. It was found that the copper content of the original tubing was quite variable (0.17 to 1.07 micrograms per 2.5 inches of tubing). The tubing was accordingly treated with 0.5 per cent H<sub>2</sub>SO<sub>4</sub> for 20 hours and with redistilled water for 4 hours to remove any copper contaminant before being used as membranes.

The contents of the membrane were washed into 100 cc. Kjeldahl flasks and digested with sulfuric and perchloric acids, followed by sodium sulfite to remove the excess perchloric acid. The

<sup>1</sup> Obtained through the courtesy of Dr. M. R. Irwin of the Genetics Department and Dr. L. C. Ferguson of the Veterinary Science Department.

copper was determined by the method of Fischer and Leopoldi (8). All reagents were extremely low in copper. The dialysates were evaporated to about 5 cc. and then digested and analyzed in the same manner.

In addition to experiments on plasma alone, various amounts of hydrochloric, sulfuric, and phosphoric acids were added to the plasma and the pH of the mixture was determined by means of a glass electrode. It was found that the pH which was obtained was quite constant for any given amount of the acid which was added. The percentage of dialyzable copper was determined with the various acids over a pH range of from 8.1 to 1.5.

### *Results*

The results are given in Table I. The pH value was obtained from a smooth curve which was constructed from experiments in which the serum was acidified with various amounts of acids of various concentrations. Table I includes all of the experiments in an arbitrary time period. A large number of preliminary experiments are not reported. It can be seen that the sum of the dialyzed and non-dialyzed copper exceeds 100 per cent by about 10 per cent, which represents about 1 microgram of copper contamination which we were unable to avoid. Table I is arranged to show the correlation between the pH of the plasma and the per cent of dialyzed copper. It also shows the relation between pH and the amount of acid which was added.

Table I shows that virtually no copper dialyzed from plasma that was not acidified. This is in agreement with the report by Abderhalden and Möller (6). The percentage of dialyzed copper increased in a direct correlation with the increase in acidity. It is interesting to note that although the pH changes produced by HCl and H<sub>2</sub>SO<sub>4</sub> were the same for given amounts of normal acid, the amount of dialyzed copper produced by the two acids was significantly different. Thus at a pH of 1.67 only 46 per cent of the copper dialyzed in the presence of HCl, while 84 per cent dialyzed in the presence of H<sub>2</sub>SO<sub>4</sub>. The work of Moore (4) was published shortly after our work was completed and showed that the amount of easily split off iron was greater with 0.2 N H<sub>2</sub>SO<sub>4</sub> than with 0.2 N HCl. At present the explanation of this difference between results with HCl and H<sub>2</sub>SO<sub>4</sub> is not known.

TABLE I  
Dialysis of Plasma Copper at Various pH Values

Sample No.	Total copper	Acid used H <sub>2</sub> SO <sub>4</sub>	pH	Dialyzed copper	Non-dialyzed copper
	<i>micrograms per 10 cc. plasma</i>	<i>gm. m.-eq.</i>		<i>per cent</i>	<i>per cent</i>
322	10.6	None	7.82	8	104
329	10.5	"	7.82	9	106
45	10.9	"	7.82	5	105
419	12.7	"	7.80	9	75
322	10.6	0.16	6.60	-3	91
329	10.5	0.16	6.60	18	101
322	10.6	0.28	5.67	9	100
329	10.5	0.28	5.67	21	99
322	10.6	0.49	4.68	30	69
329	10.5	0.49	4.68	36	83
322	10.6	0.67	4.23	48	61
329	10.5	0.67	4.23	52	71
45	10.9	0.70	4.18	28	66
45	10.9	1.04	3.43	46	61
45	10.9	1.33	2.64	64	50
45	10.9	1.69	1.99	74	46
419	12.7	1.69	1.99	59	43
419	12.7	2.08	1.68	84	38
		H <sub>2</sub> PO <sub>4</sub>			
621	12.5	None	7.82	7	105
621	12.5	0.22	6.57	16	91
621	12.5	0.75	5.43	12	97
621	12.5	3.74	2.52	37	64
621	12.5	7.48	1.92	53	42
621	12.5	11.22	1.67	78	33
		HCl			
630	12.0	None	7.85	12	92
426	11.1	"	7.82	11	90
419	12.7	"	7.80	9	75
630	12.0	0.22	6.05	6	97
426	11.1	0.35	5.35	17	83
630	12.0	0.48	4.70	28	77
426	11.1	0.66	4.20	24	90
426	11.1	1.02	3.45	31	81
630	12.0	1.05	3.40	24	90
630	12.0	1.66	2.05	45	75
419	12.7	2.10	1.67	46	60

Barkan (9) has reported that the copper from hemocyanin was split off when the blood was diluted with 0.4 per cent HCl, but there has been no evidence for a hemocyanin compound in mammalian blood. Eisler *et al.* (3) have suggested that copper is in the form of an albuminate in plasma and give this as an explanation for their results obtained with protein precipitations on the acid and on the alkaline side of the isoelectric point of serum albumin. It would seem that if their interpretation were correct the per cent of copper dialyzed would suddenly increase when the pH of the serum reached the isoelectric point of serum albumin. Our results show quite definitely that such is not the case.

Warburg and Krebs (1) did not differentiate between loosely bound and total copper; that is, they believed that all of the copper in serum was loosely bound. Our results indicate that at the acidity employed by Warburg and Krebs the copper would not be 100 per cent dialyzed. There must therefore be some question as to the validity of the concept that serum copper is 100 per cent loosely bound, although it is possible that the catalysis of cysteine is a better criterion than the dialyzability of the copper. Another explanation would be that the copper might be 100 per cent dialyzed at the lower pH values if sufficient time were allowed.

The work of Barkan and of Moore seems to make it probable that iron is absorbed in the ionic form and is first found in the blood stream as easily split off iron. From this form the iron is converted ultimately into hemoglobin. There is some question as to how close an analogy exists in the case of copper. The latter is undoubtedly absorbed as the copper ion and certainly exists at least in part in a loosely bound form. If the plasma copper is 100 per cent in this form, as the work of Warburg and Krebs suggests, then there is no balance between loosely bound and firmly bound organic copper in blood, and hence there would be no object in studying the percentage of loosely bound copper in various stages of anemia. This question should be settled before studies of loosely bound copper in anemia are made. It is also of importance to know whether or not the copper as it exists in blood plasma is capable of showing the catalytic properties of copper ions. Barron *et al.* (10) have reported that the copper catalysis of ascorbic acid oxidation is inhibited by protein, while Stotz *et al.* (11) have stated that the catalytic activity of various

ascorbic acid oxidase preparations can be explained on the basis of their copper content.

#### SUMMARY

1. Blood plasma was dialyzed in the presence of varying amounts of acid and the dialysates were analyzed for copper.

2. It was found that when no acid was added the copper did not dialyze and that increasing amounts of copper dialyzed as the pH was progressively lowered.

3. It was concluded that copper exists in plasma in an organic form which is dissociated by acid.

4. It was found that hydrochloric and sulfuric acids produced identical changes in the pH of plasma but that much more copper dialyzed in the presence of sulfuric acid.

5. The possibility that copper exists in more than one organic form in plasma is discussed.

The authors are indebted to Dr. C. A. Elvehjem for his kind interest and helpful criticism.

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## THE CONFIGURATIONAL RELATIONSHIP OF 3-AMINO-HEPTANE TO THAT OF NORLEUCINE

BY P. A. LEVENE AND MARTIN KUNA

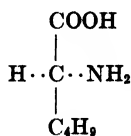
*(From the Laboratories of The Rockefeller Institute for Medical Research, New York)*

(Received for publication, October 25, 1937)

Two amines, 2-aminoheptane and 3-aminoheptane, having optical rotations of the same sign were found to display rotatory dispersions of a different type, the first having a normal, the second an anomalous course. Anomalous rotatory dispersion is an indication that the direction of rotation of a substance in the visible region is determined by the partial rotation of the absorption band located in the more distant region of the spectrum than that of the first anisotropic absorption band. It is known, on the basis of past experience, that in members of homologous series corresponding absorption bands as a rule furnish partial rotations of the same sign. It could thus be concluded that in the two amines the corresponding absorption bands furnished partial rotations of opposite sign and hence different configurations might have been assigned to the two amines. However, there are data contradicting this conclusion. First, the two amines have been prepared from two azides rotating in the same direction and derived from two corresponding iodides of the same configuration. Second, the benzoyl derivatives of the two amines rotated in the same direction and displayed dispersion curves of the same character. It was therefore urgent to arrive at a decision as to the configurational relationship of the two substances by methods of classical organic chemistry. This aim has now been accomplished by the correlation of each of the two amines to one amino acid.

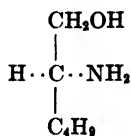
The correlation of the configuration of dextro-2-aminoheptane to that of levo- $\alpha$ -aminocaproic acid has been accomplished by the reduction of the ester of the amino acid to norleucinol and of the

latter to the amine. Thus, the configuration of norleucinol is correlated both to  $\alpha$ -aminocaproic acid and to 2-aminoheptane, as in Formulæ I, II, and III.



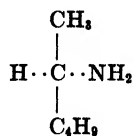
I

Levo



II

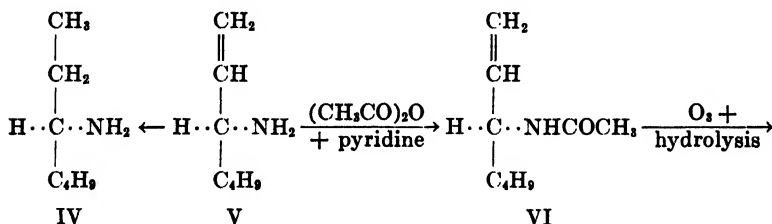
Levo



III

Dextro

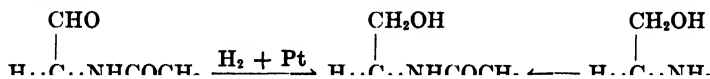
Hence, if the configuration of 3-aminoheptane could be correlated to that of norleucinol, then its correlation to that of  $\alpha$ -aminocaproic acid and of 2-aminoheptane should follow automatically. This task has been accomplished by a set of relationships given in Formulæ IV to IX. From the formulæ it can be seen that the acetyl derivatives were used throughout the experiments. This was done for convenience of operation.



Dextro

Levo

Levo

C<sub>4</sub>H<sub>9</sub>

VII

Dextro

C<sub>4</sub>H<sub>9</sub>

VIII

Dextro

C<sub>4</sub>H<sub>9</sub>

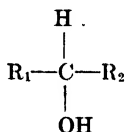
IX

Levo

Thus the configuration of dextro-3-aminoheptane is correlated to that of dextro-2-aminoheptane and the configurations of both amines are correlated to that of levo- $\alpha$ -aminocaproic acid.

The configuration previously assigned to 2-aminoheptane on the basis of the anomalous course of its rotation should be revised.

A rigorous explanation for the differences in the rotatory dispersion of the two substances cannot be given at this date for the reason that the distinctly anisotropic bands of the amines are located in the distant ultraviolet region of the spectrum. An explanation, however, can be offered postulating that the rotatory dispersion of the 3-aminoheptane is quasi normal, the partial contribution of the nearest anisotropic band being negligible in value. A similar assumption has been made in order to explain the differences in the rotatory dispersions in members of series of secondary carbinols of the type



homologous with respect to  $\text{R}_1$ .

#### EXPERIMENTAL

*Levo-3-Acetamino-1-Heptene*—31 gm. of 3-amino-1-heptene,<sup>1</sup>  $\alpha_D^{25} = -4.80^\circ$  (homogeneous, 1 dm.), were dissolved in 36 gm. of dry pyridine, and were slowly added to 90 gm. of acetic anhydride which was cooled in a freezing mixture. The solution was allowed to stand overnight at  $20^\circ$  and was then poured into water and extracted with ether. The extract was washed with dilute sulfuric acid until the washings were acid and then with dilute carbonate solution until the washings were alkaline. The extract was then dried with drierite. The substance distilled at  $105\text{--}110^\circ$ ,  $p = 1.5$  mm. Yield 25 gm.  $d_4^{25} = 0.8950$  (*in vacuo*).  $n_D^{25} = 1.4577$ .

$$[\alpha]_D^{25} = \frac{-0.67^\circ}{1 \times 0.89} = -0.75^\circ; \quad [M]_D^{25} = -1.16^\circ \text{ (homogeneous)}$$

4.533 mg. substance: 11.605 mg.  $\text{CO}_2$  and 4.430 mg.  $\text{H}_2\text{O}$

$\text{C}_9\text{H}_{17}\text{ON}$ . Calculated. C 69.61, H 11.05  
155.1 Found. " 69.81, " 10.94

*Dextro-2-Acetaminohexaldehyde*—25 gm. of 3-acetamino-1-heptene,  $[\alpha]_D^{25} = -0.75^\circ$  (homogeneous), were dissolved in 100 cc.

<sup>1</sup> Levene, P. A., Rothen, A., and Kuna, M., *J. Biol. Chem.*, **120**, 777 (1937).



of glacial acetic acid and ozonized in four lots. The ozonide was decomposed as usual, and the ether solution was neutralized by adding powdered potassium carbonate, and a little water. The ether was decanted and dried with anhydrous potassium car-

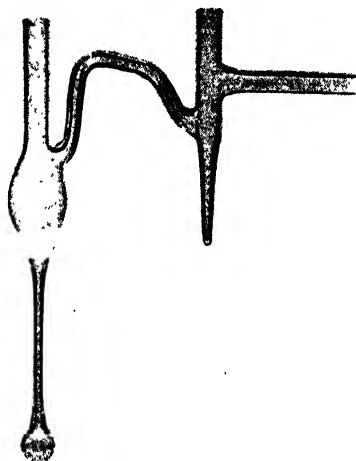


FIG. 1. Microdistilling flask. The diameter of the bottom bulb is 12 mm. and the capillary tubing is 1.5 mm. inside diameter. The flask is immersed up to the delivery tube in a mineral oil bath equipped with a mechanical stirrer as recommended by Craig.<sup>2</sup> Various sizes were made, and were found useful in the distillation of very viscous materials, such as sugar derivatives.

bonate. The aldehyde distilled at 130–135°,  $p = 3$  mm. Yield 6 gm.

$$\alpha_D^{25} = +6.23^\circ \text{ (homogeneous, 1 dm.)}$$

$$[\alpha]_D^{25} = \frac{+0.72^\circ \times 100}{1 \times 12.7} = +5.7^\circ; \quad [M]_D^{25} = +8.9^\circ \text{ (in ether)}$$

3.908 mg. substance: 8.600 mg. CO<sub>2</sub> and 3.432 mg. H<sub>2</sub>O

C<sub>8</sub>H<sub>15</sub>O<sub>2</sub>N. Calculated. C 61.09, H 9.63

157.1 Found. " 60.01, " 9.82

<sup>2</sup> Craig, L. C., *Ind. and Eng. Chem., Anal. Ed.*, **8**, 219 (1936).

*Dextro-2-Acetamino-1-Hexanol* (*Acetylnorleucinol*)—3 gm. of 2-acetaminohexaldehyde,  $\alpha_D^{25} = +6.23^\circ$  (homogeneous, 1 dm.), were dissolved in 50 cc. of dry ether, and 0.5 gm. of Adams' catalyst was added. This was shaken with hydrogen at a pressure of 3 atmospheres for 36 hours. The catalyst was filtered off, and the ether was evaporated from the filtrate. The residue was distilled from a special microdistilling flask (Fig. 1). The bath temperature was 150–165° and the pressure 0.1 mm. Yield 1 gm.

$$[\alpha]_D^{25} = \frac{+0.43^\circ \times 100}{1 \times 12} = +3.6^\circ; \quad [M]_D^{25} = +5.7^\circ \text{ (in absolute alcohol)}$$

4.510 mg. substance: 9.976 mg. CO<sub>2</sub> and 4.365 mg. H<sub>2</sub>O

C <sub>8</sub> H <sub>17</sub> O <sub>2</sub> N. Calculated. C 60.32, H 10.77		
159.1	Found.	" 60.32, " 10.83

*Dextro-2-Acetamino-1-Hexanol* (from *Norleucinol*)—3 gm. of norleucinol hydrochloride,<sup>3</sup>  $[\alpha]_D^{24} = -0.95^\circ$  (in water), were dissolved in methanol and 16 cc. of 1.36 N sodium methylate were added. This was cooled, and the precipitated salt was filtered off. Ketene was passed into the filtrate until its reaction was slightly acid. The solution was then concentrated and the residue was distilled from a microflask. The bath temperature was 135–150°,  $p = 0.1$  mm. Yield 0.2 gm.

$$[\alpha]_D^{25} = \frac{+0.32^\circ \times 100}{1 \times 15} = +2.1^\circ; \quad [M]_D^{25} = +3.3^\circ \text{ (in absolute alcohol)}$$

4.022 mg. substance: 8.861 mg. CO<sub>2</sub> and 3.850 mg. H<sub>2</sub>O

C <sub>8</sub> H <sub>17</sub> O <sub>2</sub> N. Calculated. C 60.32, H 10.77		
159.1	Found.	" 60.07, " 10.71

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<sup>3</sup> Levene, P. A., and Mardashew, S., *J. Biol. Chem.*, **117**, 707 (1937).



## ACTIVATION OF TESTOSTERONE BY HIGHER FATTY ACIDS AND THEIR ACID SODIUM SALTS\*

BY MAXIMILIAN EHRENSTEIN AND EDWARD L. COREY

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(Received for publication, October 1, 1937)

The influence of male sex hormones on the development of the accessory sex organs in the rat can be enhanced: (a) by esterification with certain lower fatty acids (1, 2); (b) by administering the hormone together with either certain higher fatty acids (3) or high molecular weight saturated primary alcohols ((3) p. 1974); the injection of the hormone and the second component must be made at the same time and site to obtain the desired effect. It has further been found that the free hormone only can be activated, and that a combination of hormone esters with fatty acids appears to bring about no further increase of the physiological effect ((1) p. 1986, (2)).

Laqueur and his collaborators found that an activating factor, the so called X substance, is present in various tissues (4). Tschopp and coworkers succeeded in isolating palmitic acid from the X substance fraction of testes by means of the chromatographic adsorption technique (5). They also investigated the activating effect of a number of naturally occurring fatty acids and found palmitic acid to be the most effective (3).

In an earlier paper Ehrenstein and Britton (6) describe the isolation of an acid sodium salt of palmitic acid,  $C_{15}H_{31}COOH \cdot C_{15}H_{31}COONa$ , from adrenal glands. Its possible action as an activator of male sex hormones is there discussed. Evidence has now been obtained that this salt is not originally present in the adrenal tissues. It is apparently formed by the permutit treatment employed during the preparation of corticoadrenal

\* Grateful acknowledgment is made of aid received from the Rockefeller Foundation.

extract. Moreover, it was observed that permutit can generally be used for the isolation of higher fatty acids from various kinds of tissue. Thus we obtained the acid sodium salt and, by acidification, palmitic acid from testes and liver. Only traces could be isolated from ovarian tissue. This indicates that the occurrence of free palmitic acid, like that of the X substance, is not restricted to the tissues of the endocrine glands.

The present paper deals especially with the activating effect of palmitic and stearic acids and their corresponding acid sodium salts on the male sex hormone testosterone.

#### EXPERIMENTAL

##### *Chemical Procedures*

*Isolation of Palmitic Acid from Various Tissues*—All tissues were treated according to the modified method used in this laboratory for the preparation of corticoadrenal extract. Before the treatment with permutit the alcoholic solution was brought to dryness. On addition of pure acetone to the residue, a sticky precipitate was obtained, apparently consisting mainly of those last traces of phospholipids which had escaped the first precipitation. On acidification of this resinous material, which contained but traces of sodium, no free fatty acid could be isolated. The acetone solution was brought to dryness, the residue dissolved in 70 per cent alcohol, and this alcoholic solution treated with permutit in the usual manner. This solution was then brought to dryness and the residue treated with acetone. In all experiments (beef adrenals, testes, ovaries, liver) a more or less powdery, light brown precipitate was obtained which, on being treated with water, yielded a white precipitate of microscopically fine flat needles. These needles represented the acid sodium salt of palmitic acid,  $C_{15}H_{31}COOH \cdot C_{15}H_{31}COONa$ , in a more or less pure form. On acidification, the free acid was obtained and could be readily identified as palmitic acid. A satisfactory yield was obtained from adrenal glands; testes and liver yielded a little less and not quite pure palmitic acid. Only traces of rather impure palmitic acid could be secured from ovarian tissue.

*Preparation of Sodium Bipalmitate*,  $C_{15}H_{31}COOH \cdot C_{15}H_{31}COONa$ , and *Sodium Bistearate*,  $C_{17}H_{35}COOH \cdot C_{17}H_{35}COONa$ —0.02 mole of pure acid (Kahlbaum) was dissolved in 80 cc. of warm 95 per

cent alcohol. To this solution was added rather quickly a warm solution of 0.01 mole of sodium hydroxide in 57 cc. of alcohol (the NaOH is first dissolved in 2 cc. of water, then 55 cc. of 95 per cent alcohol are added). On standing, the salt crystallized in long flat spears. The melting point of the bipalmitate was 122–124° (sintering between 80–90°) and that of the bistearate 121–124° (sintering at about 100°). It should be noted that the molten salts were not quite transparent.

### *Animal Experiments*

*Method of Assay*—Young, male white rats weighing 50 to 80 gm. were castrated. They were used for assaying the various preparations not before the 25th day and not later than the 30th day after operation. Subcutaneous injections were carried out for 9 successive days, and sacrifice of the animals and dissections were made on the 10th day. The daily dose was always dissolved in 0.5 cc. of sesame oil. In those cases in which testosterone was combined with either a free acid or an acid sodium salt, the two constituents were dissolved in 0.25 cc. of sesame oil each. The syringe was filled by drawing in the solution of testosterone first and that of the activator thereafter. Since palmitic acid and stearic acid as well as their corresponding acid sodium salts are only slightly soluble in sesame oil at room temperature, these preparations were heated in a paraffin bath to about 130° (*i.e.* a little higher than the melting points of the salts). Although the free acids yield a homogeneous solution at a considerably lower temperature, they were heated as high as the salts in order to insure comparable conditions. It should be stated that with the higher concentrations of acid, and especially of salt, a formation of lumps under the skin was observed. The health of the rats was not, however, impaired; all experimental animals appeared to be in good condition at the time of sacrifice.

### *Results*

By comparison of the results in Tables I and II, it will be seen that under the experimental conditions described, the effect of testosterone on the development of the seminal vesicles and prostate gland can be considerably enhanced by combining the hormone either with free higher fatty acids (palmitic acid, stearic

acid) or their corresponding acid sodium salts ( $C_{15}H_{31}COOH$ .- $C_{15}H_{31}COONa$ ,  $C_{17}H_{35}COOH$ .- $C_{17}H_{35}COONa$ ). Rather low doses (up to 25 mg. daily) of the salts proved to be much more effec-

TABLE I  
*Activation of Testosterone*

50 micrograms of testosterone and various concentrations of activator were administered daily to castrated rats.

No. of rats	Concentration and kind of activator	Average body weight at killing	Average weight		Seminal vesicles	Prostate
			Seminal vesicles	Prostate		
Palmitic acid						
	<i>mg.</i>	<i>gm.</i>	<i>mg.</i>	<i>mg.</i>	<i>per cent of body weight</i>	<i>per cent of body weight</i>
4	25 Palmitic acid	145	51	46	0.034	0.031
4	50 " "	156	154	86	0.100	0.055
4	10 Na bipalmitate	146	92	66	0.060	0.045
6	25 " "	135	139	75	0.104	0.055
6	50 " "	126	185	96	0.147	0.075
	Standard deviation*..	12.1	36.5	14.6	0.0293	0.0112
Stearic acid						
4	25 Stearic acid	158	70	45	0.044	0.028
4	50 " "	166	175	100	0.106	0.061
4	75 " "	177	311	121	0.176	0.068
4	10 Na bistearate	176	110	49	0.062	0.028
4	25 " "	156	157	89	0.102	0.057
4	50 " "	153	144	87	0.095	0.057
4	75 " "	152	135	91	0.099	0.060
	Standard deviation*..	9.2	41.0	11.4	0.0263	0.0072

The figures contained in the table confirmed preliminary experiments carried out with fewer animals.

\* Pooled standard deviation of observations within groups from their respective means.

tive than the same doses of the free acids. With medium doses (50 mg. daily) the effect was about the same with either the free acid or the acid sodium salt. Higher doses of free acid (75 mg. daily), as shown in the stearic acid series, brought about a further

increase of the activating effect. Activation with the acid sodium salt, however, could not be increased beyond that obtained with the 50 mg. daily dosage.

TABLE II

*Effectiveness of Testosterone, Testosterone Acetate,\* and Testosterone Propionate†*

Castrated rats were given 50 micrograms of the various preparations daily, no activator being added.

In the case of control animals which were given only 0.5 cc. of pure sesame oil daily, the average weight of the seminal vesicles was 5 mg. and of the prostate 3.9 mg. (2 animals).

No. of rats	Concentration of male hormone in 0.5 cc. sesame oil	Average body weight at killing	Average weight		Seminal vesicles	Prostate
			Seminal vesicles	Prostate		
		gm.	mg.	mg.	per cent of body weight	per cent of body weight
5	Normal animals; no injection given	150	205	110	0.134	0.072
	Standard deviation‡		67.1	34.7	0.0347	0.0182
4	50 micrograms testosterone	140	25	21	0.018	0.016
3	50 " " acetate	164	83	54	0.056	0.037
4	50 micrograms testosterone propionate	158	62	34	0.041	0.021
	Standard deviation‡	20.4	21.2	9.0	0.0197	0.0066

\* Perandren, a preparation of the Ciba Company, Inc., New York. By courtesy of Dr. Haskell.

† Testoviron, a preparation of the Schering-Kahlbaum, A. G., Berlin. By courtesy of Professor W. Schoeller.

‡ Pooled standard deviation of observations within groups from their respective means.

A satisfactory explanation of the action of the salts cannot be given at this time. Possibly the suspension of the salt in sesame oil, which represents a mass of vaseline-like consistency, acted as a colloidal system such that if 25 mg. or more of the salt was administered daily all of the testosterone was adsorbed by it and slowly liberated at a more or less constant rate.



The activity of highly effective esters of testosterone (such as the acetate and propionate) was considerably surpassed in these experiments. Furthermore, the weights of the accessory sex organs of normal animals of approximately the same body weight were exceeded in the experiments in which 50 micrograms of testosterone were combined with 75 mg. of free acid.

It is known that testosterone palmitate and stearate ((1) p. 1980) are almost inactive. Thus the suggestion of an ester theory for the explanation of the activating effect of free higher fatty acids is not justified.

#### SUMMARY

The increased effect of testosterone upon the accessory sex organs of the male rat induced by palmitic or stearic acid or by their acid sodium salts is demonstrated by comparison with the effect of testosterone alone or with that of two esters of testosterone.

The authors feel much indebted to Dr. Erwin Schwenk of the Schering Corporation, Bloomfield, New Jersey, for the generous supply of testosterone.

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## THE ISOLATION OF ESTROGENIC DIOLS FROM THE URINE OF PREGNANT MARES

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In 1932 Schwenk and Hildebrandt reported the isolation of a new estrogenic substance from the urine of pregnant mares (1). This substance, the melting point of which was given as 200–209°, was designated  $\delta$ -follicular hormone by the authors. In contrast to the other estrogenic principles isolated from this source, estrone, equilin, equilenin, and hippulin, it lacked ketonic properties. Subsequently Wintersteiner, Schwenk, and Whitman (2) showed that both oxygen atoms of this compound were present as hydroxyl groups. Last year larger amounts of starting material were obtained through the courtesy of Dr. Erwin Schwenk of the Schering Corporation, who invited us to attempt a further elucidation of the chemical nature of this compound.

The non-ketonic phenolic fraction after removal of contaminating substances by distribution procedures and high vacuum distillation was purified by repeated recrystallization. The final product showed a constant melting point of 226°; that is, 15° higher than that of the best previous preparation. It did not change in composition on fractional high vacuum distillation. These facts as well as the intensity of the characteristic color reactions (1, 3) led us to believe that the preparation in our hands was the pure  $\delta$ -follicular hormone. On analysis the new substance gave figures approximating the values required by the formula  $C_{18}H_{22}O_2$ , in agreement with the findings of Schwenk *et al.* for their original

\* Columbia University Fellow, 1937–38. This report is from a dissertation submitted by H. Hirschmann in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

preparation (1).<sup>1</sup> Ultraviolet absorption measurements, however, indicated the inhomogeneity of this crystalline substance melting at 226°. The spectrum (Curve 1, Fig. 1) differed markedly from those of the other known estrogens. Four of the seven maxima encountered in the spectrum appear also in the spectrum of equilenin as recorded by Dirscherl and Hanusch (5), but their intensi-

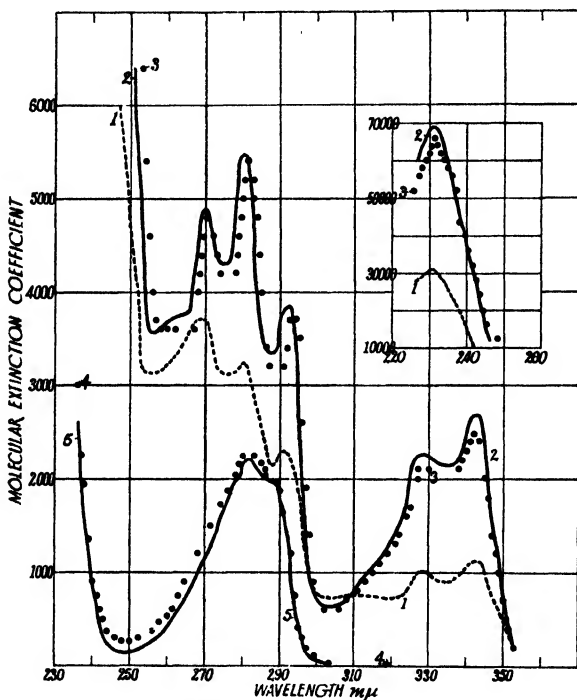


FIG. 1. Absorption spectra. Solvent, 95 per cent alcohol. Curve 1 represents the crystals melting at 226°; Curve 2, dihydroequilenin; Curve 3, equilenin (preparation of Dr. A. Girard); Curve 4,  $\beta$ -estradiol isolated from urine; Curve 5,  $\beta$ -estradiol obtained by reduction of estrone.

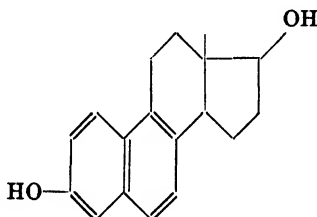
ties showed no correlation at all with those of the maxima in the equilenin spectrum. In particular the maximum at 343  $m\mu$ ,

<sup>1</sup> The formula  $C_{18}H_{24}O_2$  which was reported in the second publication (2) is apparently responsible for the confusion regarding the structure of  $\delta$ -follicular hormone prevailing in the review literature (4). It was never intimated that it might be identical with the diol now designated  $\beta$ -estradiol.

located in a range where no other estrogen but equilenin absorbs light, was about twice as high in the equilenin spectrum as in that of the new substance. In an attempt to secure more of this product from the mother liquors a fraction was obtained, the melting point of which could not be raised beyond  $217^{\circ}$ , although in respect to crystal form, color reactions, and solubility properties it appeared similar to the substance melting at  $226^{\circ}$ . Spectrographic analysis revealed a spectrum closely approximating that of equilenin in location as well as in the intensity of the bands. This suggested that the higher melting product was inhomogeneous and contained the equilenin-like substance melting at  $217^{\circ}$  as one of its components. This assumption proved to be correct, as it was found that fractionation occurred on distribution between alkali and ether; the substance exhibiting the equilenin spectrum accumulated in the alkaline layer. Furthermore, only this fraction yielded an insoluble picrate in alcoholic solution. In the subsequent work isolation of the product melting at  $226^{\circ}$  was abandoned and the purified non-ketonic phenols were directly subjected to fractionation by the above methods. Spectrographic analysis proved to be indispensable in the course of this fractionation, since melting point data were almost useless for controlling the purity of the various fractions. Not only do all three products finally isolated melt within a few degrees, but also the melting points of mixtures lie in approximately the same range. This is probably due to the formation of solid solutions and, as has been proved in one instance, of a molecular compound. Also, the spectrographic method gave more specific information qualitatively as well as quantitatively than rotation measurements. While the distribution between sodium hydroxide and an organic solvent (6) and the purification through the picrate (7) proved equally useful, as in the work with the ketonic hormones, fractional crystallization (8) was of little avail. Only the equilenin-like substance, the chief component of our mixtures, could in some instances be obtained in fairly pure state by direct recrystallization. The purification of the other two substances by fractional crystallization proved impracticable.

*17-Dihydroequilenin*—The material accumulating in the alkaline phase in the distribution mentioned above was purified by way of the picrate. The final product after recrystallization showed a

constant melting point of  $217^{\circ}$ . The color reactions originally described as characteristic for the  $\delta$ -hormone are given by the new compound. The analysis corresponded to  $C_{18}H_{20}O_2$ , which is the composition of a dihydroequilenin. Both oxygen atoms are present as hydroxyl groups, as ascertained by the formation of a diacetate and a di-*p*-nitrobenzoate. Its spectrum (Curve 2, Fig. 1) is identical with that of equilenin (Curve 3, Fig. 1). Incidentally, our measurements on equilenin reveal the presence of two bands in addition to those recorded by Dirscherl and Hanusch (5); namely, at 231 and 293  $m\mu$ . The above observations are in accordance with the view that this new diol is a 17-dihydro-equilenin.



Proof of this structure was adduced by chromic acid oxidation of the 3-monobenzoate to a benzoylated ketone identical with equilenin benzoate. The proposed structure received additional confirmation by the work of Marker, Kamm, Oakwood, and Tenedick (9) published subsequently to our preliminary report (10). These authors reduced equilenin with aluminum isopropylate and obtained two epimeric diols, the lower melting of which possesses the properties of the substance isolated by us.

*$\beta$ -Estradiol*<sup>2</sup>—The material which accumulated in the ether phase, after removal of various impurities by distribution methods, yielded a product melting at  $224^{\circ}$ . The analytical figures indicated a formula  $C_{18}H_{24}O_2$ . The 2 oxygen atoms are present as hydroxyl groups. The spectrum closely resembled that of estrone (12). We, therefore, concluded that we were dealing with an estradiol. However, the melting point of our compound precludes its identity with the estradiol (melting at  $178^{\circ}$ ) isolated from follicular fluid by MacCorquodale, Thayer, and Doisy (13)

<sup>2</sup> Announcement of the isolation of  $\beta$ -estradiol has been made (11).

and from mare urine by Wintersteiner, Schwenk, and Whitman (2). The estradiol melting at  $178^{\circ}$  can also be obtained by reduction of estrone. However, Schwenk and Hildebrandt (14), who first carried out this reduction, also observed the formation of an isomer melting at  $204^{\circ}$ . Since it seemed highly probable that the estradiol isolated from urine was identical with the high melting diol of Schwenk and Hildebrandt, the reduction of estrone was reinvestigated by Whitman, Wintersteiner, and Schwenk (15). The high melting diol obtained in pure state by these authors melted at  $223^{\circ}$ . The preparation of the same compound was later accomplished independently by Butenandt and Goergens (16). The name  $\beta$ -estradiol was assigned to the isomer to distinguish it from the isomer melting at  $178^{\circ}$  ( $\alpha$ -estradiol). The identity of the estradiol from mare urine with the  $\beta$ -estradiol obtained by reduction of estrone was established beyond doubt by comparison of rotation, color reactions, and the melting points of the two products, as well as of their diacetate and monobenzoate. In view of the complete agreement of these physical properties it was somewhat surprising that a slight but distinct discrepancy existed between the ultraviolet spectra of the two preparations. The difference is especially apparent at the wavelength of the minimum ( $249\text{ m}\mu$ ) where the compound from urine showed stronger absorption than the synthetic product. Also, the former exhibited some absorption between 305 and  $325\text{ m}\mu$ , a range where the synthetic compound absorbed no light. Obviously the natural preparation still contained small amounts of a contaminating substance with characteristic spectroscopic properties.<sup>3</sup> Removal of this contaminant was finally accomplished by chromatographic analysis. Even after this treatment a slight difference between the spectra of the final product and of the synthetic estradiol was still apparent (Curves 4 and 5, Fig. 1), but the amount of contaminant present in the former was estimated as only about 1 per cent.

*Molecular Compound of Dihydroequilenin and  $\beta$ -Estradiol*

*Isolation of a Third Compound Isomeric with Dihydroequilin—*  
By the methods designed for the fractionation of the non-ketonic

<sup>3</sup> We believe that the absorption in this range recorded by Mayneord and Roe (17) for estrone is likewise due to a—possibly related—impurity.

phenolic fraction the product originally isolated by direct crystallization (m.p.  $226^{\circ}$ ) can be separated into dihydroequilenin (m.p.  $217^{\circ}$ ) and  $\beta$ -estradiol (m.p.  $224^{\circ}$ ). The melting point data indicate that the product obtained by direct crystallization is a molecular compound of dihydroequilenin and  $\beta$ -estradiol. Indeed, on recrystallizing together equivalent amounts of the latter two compounds apparently homogeneous needles were obtained which melted even higher than the crystalline product first obtained from urine; namely, at  $227-228.5^{\circ}$ . The discrepancy indicated that the crystals from urine did not have the exact composition of the molecular compound, and this is also borne out by certain characteristics of its spectrum. Superimposition of the curves of dihydroequilenin and  $\beta$ -estradiol does not reproduce the experimental curve (Fig. 1) no matter what ratios of the two compounds are taken. Especially the fact that the substance melting at  $226^{\circ}$  absorbs more strongly at  $270\text{ m}\mu$  than at  $280\text{ m}\mu$ , whereas the opposite is true for the two isolated components, led us to suspect the presence of a third substance with an absorption maximum between 260 and  $270\text{ m}\mu$ . With the help of this spectroscopic lead it was possible to isolate the unknown compound, here provisionally designated Compound 3. The best source for its isolation was found to be the filtrate from the dihydroequilenin picrate, in which, however, considerable amounts of dihydroequilenin and  $\beta$ -estradiol were still present. No method could be devised by which both these compounds could be separated from Compound 3 simultaneously. Chromatographic analysis served well for the removal of  $\beta$ -estradiol but was ineffective for the elimination of dihydroequilenin. The latter had to be removed by distribution procedures. As unavoidably great losses were incurred in all these steps, only 4 mg. of white needles, melting at  $223.5^{\circ}$ , were finally isolated. The spectrum (Fig. 2), showing a very intense band at  $264\text{ m}\mu$  and a rather indistinct low band at  $292\text{ m}\mu$ , is different from those of any of the well characterized estrogens. The product still contained about 8 per cent dihydroequilenin. In spite of this contamination the analysis agreed well with  $\text{C}_{18}\text{H}_{20}\text{O}_2$ . Calculation shows that the presence of 8 per cent dihydroequilenin could affect the carbon and hydrogen figures by less than 0.06 per cent. Lack of material prevented the characterization of the 2 oxygen atoms. One of them must be

present as a phenolic hydroxyl. The mode of isolation and the great similarity of the physical and chemical properties of Compound 3 with those of the two other compounds isolated make it probable that the 2nd oxygen atom is likewise hydroxylic. The spectroscopic properties clearly indicate the presence of a double bond in Ring B in conjugation to the benzenoid ring; that is, either between carbon atoms 6 and 7 or 8 and 9. This concept is supported by comparison with the spectrum of 1,2-dihydronaphtha-

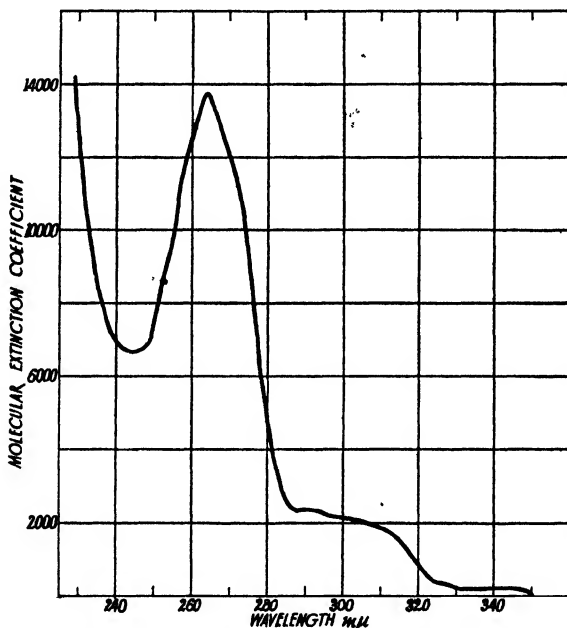


FIG. 2. Absorption spectrum of Compound 3 in 95 per cent alcohol

lene (maxima at 262 and 296 mμ) (18). Of the known ketonic estrogens which possess four ring double bonds, equilin (19), isoequilin (20), and hippulin (19), only the first two have been investigated spectroscopically. The spectrum of equilin is identical with that of estrone; that of isoequilin exhibits maxima at 265, 275, and 334 mμ. Inhoffen proposes two alternative structures for isoequilin which are analogous to those suggested above for Compound 3. On the basis of the spectroscopic data it would appear



that the new compound corresponds structurally to the alternative bond isomer of isoequilen rather than to isoequilen itself.

### Bioassays<sup>4</sup>

The results of the bioassays are given in Table I.

Our present preparation of  $\beta$ -estradiol from urine assayed for 50,000 to 75,000 rat units per gm., while the activity of the compound prepared by reduction of estrone was given as 300,000 rat units per gm. (15). It is highly probable that the discrepancy is due to the presence of a small amount (2 per cent) of the highly active  $\alpha$  epimer in the synthetic preparation. Since Compound 3 was still contaminated with about 8 per cent of dihydroequilenin, it is doubtful whether this substance has any estrogenic activity of its own. The amount available was insufficient to decide this question.

TABLE I  
*Physiological Activity in Rat Units per Gm.*

Diol isolated from urine	C <sub>17</sub> epimer	Corresponding ketone (C <sub>17</sub> )
Dihydroequilenin 100,000	$\alpha$ -Estradiol 12,000,000	Equilenin 180,000
$\beta$ -Estradiol 75,000		Estrone 1,000,000
Compound 3 10,000		

### Stereochemical Relations

The possible stereochemical relationship of  $\alpha$ -estradiol to 17-*trans*-testosterone and of  $\beta$ -estradiol to the 17-*cis*-testosterone (21) has been discussed in previous publications from this laboratory (15, 22) and also by Butenandt and Goergens (16). The dihydroequilenin from mare urine cannot be placed with certainty in either stereochemical series, since its epimer, which has been described by Marker *et al.* (9), is as yet not characterized in regard to certain pertinent properties such as optical rotation and physiological activity. Our dihydroequilenin, which is identical with the lower melting of Marker's epimers, is less potent physiologically than the corresponding ketone, equilenin. This fact

<sup>4</sup> The assays were conducted by Dr. C. Mazer of Philadelphia. The details of the bioassay method have been given in a previous publication (15).

would seem to place the compound in the *cis* or  $\beta$  series of C<sub>17</sub> epimers. Also, the reaction with digitonin (23), by which  $\alpha$ -estradiol can be readily separated from its epimer, would seem to offer a possibility of distinguishing the two stereochemical series. Our dihydroequilenin does not precipitate with digitonin, which suggests a possible stereochemical relationship to  $\beta$ -estradiol. However, the fact that neither *trans*- nor *cis*-testosterone forms an insoluble digitonide invalidates this argument.<sup>5</sup> Obviously the  $\alpha$  configuration of C<sub>17</sub> is a necessary but not sufficient condition for the formation of insoluble digitonides of this type. The melting points of our dihydroequilenin and its diacetate are lower than those reported by Marker *et al.* for the epimeric compound. This is contrary to what should be expected from the biological data, since in the case of the estradiols it is the lower melting epimer which is more active than the ketone.

*Possible Occurrence of Other Diols in Urine of Pregnant Mares*—Since our ketonic fraction contained equilin as well as equilenin and estrone, it was somewhat surprising that no evidence for the presence of its reduction product, dihydroequilin, was obtained. The spectroscopic method is unsuitable for the detection of this compound, since the equilin spectrum is identical with that of estrone. On the other hand, measurement of the optical rotation should have given a clue as to its presence. The rotation of the dihydroequilin prepared by David (24), which is presumably the  $\alpha$  epimer, is  $+220^\circ$ , as compared with that of  $\alpha$ -estradiol ( $+81^\circ$ ), and a similar relationship is likely to obtain between the rotation of  $\beta$ -dihydroequilin and  $\beta$ -estradiol. Rotation measurements on suitable fractions, however, gave no evidence for the occurrence of a compound with considerably higher dextrorotation than that of  $\beta$ -estradiol.

The original preparations of  $\delta$ -hormone and its derivative were undoubtedly mixtures containing all three diols described in this paper. The color reactions ascribed to the  $\delta$ -hormone are given only by dihydroequilenin; the other properties reported differ markedly from those of our present compounds. Special mention, however, must be made of the high physiological potency which Schwenk and Hildebrandt reported for their  $\delta$ -hormone.

<sup>5</sup> The experiment with *cis*-testosterone was carried out by Professor Ruzicka, to whom we are very much obliged for this information.

Their preparations assayed about twice as high as "other potent crystallizates from pregnant mares' urine," presumably estrone, while each one of our three compounds is much less active than estrone. The high activity of Schwenk's preparation may possibly have been due to the presence of the highly potent  $\alpha$ -estradiol, which was later (2) actually isolated in small amounts from  $\delta$ -hormone fractions. We have made repeated but always unsuccessful attempts to isolate  $\alpha$ -estradiol from our material by means of the digitonin reaction. This failure of our present material to yield  $\alpha$ -estradiol may be due to variations in the composition of the original source, or to the introduction of certain modifications in the preparations of the crude extract. One of these was the use of magnesium oxide as an adsorbing agent, which undoubtedly effected fractionation of the diols.<sup>6</sup>

#### EXPERIMENTAL

Our starting material was obtained from about 20,000 liters of urine of mares in the 6th to 9th month of pregnancy, with occasional inclusion of urine of the 4th and 5th months. The conjugated estrogens were saponified by acid and alkaline hydrolysis; the alkaline solution was acidified with carbon dioxide and extracted with ether. The product was once recrystallized and then adsorbed on magnesium oxide from a dioxane-ether solution. It had been observed that the material giving the color reactions of the  $\delta$ -hormone could be separated from other estrogens in this way. All these operations were devised and carried out in the laboratories of the Schering Corporation. The eluate of the magnesium oxide adsorption was kindly supplied to us by Dr. Schwenk.

9.88 gm. of this crude phenolic fraction were separated into ketonic and non-ketonic fractions by means of 12.1 gm. of Girard's Reagent T (betaine hydrazide). The procedure used by Girard and Sandulesco (26) was followed in detail. In the extractions rather stable emulsions formed; these were broken by centrifuging. In this way large amounts were removed of a brown amorphous material which was insoluble in either phase. This insoluble

<sup>6</sup> While this paper was in press, a preliminary communication (August, 1937) by van Stolk and de Lenchere (25) came to our attention, in which the isolation of surprisingly high amounts of  $\alpha$ -estradiol (20 gm. from 15 tons of urine of pregnant mares) is reported.

material was once extracted with ether, and the extract was combined with the chief ether fraction containing the non-ketonic compounds. This ethereal solution (about 600 cc.) was once washed with water and then with 500 cc. of 8 per cent sodium carbonate in six portions. In this way 0.23 gm. of a dark brown oil was removed. The ether phase after washing with water and drying with sodium sulfate yielded 3.25 gm. of a red oil. The aqueous layer in the Girard separation gave 5.09 gm. of brown crystals (ketonic fraction).

Since the non-ketonic fraction obtained above was not free from ketones, it was treated with another ketone reagent, namely carboxymethoxylamine (0.75 gm.). We followed closely the directions of Anchel and Schoenheimer (27), who first used this reagent for the separation of ketonic sterols. The reason for the employment of the latter ketone reagent was that the non-ketonic fraction appeared to be less pigmented than that resulting from the Girard process. For the isolation of estrogenic ketones, however, Girard's reagent seems to be preferable. The extraction of the ethereal solution with potassium carbonate, which is used in the second procedure to separate the ketones from the non-ketonic material, again removed considerable amounts of pigments which apparently had formed during the reaction. The resulting non-ketonic fraction—a red, partly crystalline material—weighed 2.65 gm. and assayed for 5000 rat units per gm. in the Allen-Doisy test. This value, however, is much too low in comparison with the amount of active material isolated in pure state.<sup>7</sup>

*Purification of Non-Ketonic Fraction*—It might be pointed out here that the work described in the following was necessarily of an exploratory character and that the methods used could probably be simplified to some extent. The non-ketonic fraction was freed from colored contaminants by an extraction procedure rather than by high vacuum distillation, as new formation of pigments seems to occur during the latter operation. The material was dissolved in 2 per cent sodium hydroxide solution and extracted with small portions of a mixture of benzene and petroleum

<sup>7</sup> When sesame oil was added to the acetone solution of an aliquot of this fraction, a fine precipitate formed which was not separated but was injected in suspension. It is possible that this precipitate rendered ineffective a part of the hormone present.

ether (4:1) until most of the red pigment (0.20 gm.) had been extracted. After the addition of an equal volume of water the alkaline layer was extracted five times with ether. (The volume used for each extraction should be at least 3 times that of the aqueous phase.) The ether contained most of the hormones (2.18 gm.), whereas the bulk of the brown pigments and other more acidic products were retained in the alkaline phase.

*Fractionation*—The purified non-ketonic phenols were subjected to a 4-fold distribution between ether and alkali. 200 cc. of peroxide-free ether and 120 cc. of 2 per cent sodium hydroxide were employed for each distribution. The alkaline phase resulting from the first distribution was reextracted with three more portions of ether which were kept separate. This alkaline extract, which contains most of the pigments, yielded 565 mg. (*Fraction A<sub>1</sub>*). The four ether solutions were then washed in the original order with 120 cc. of alkali, and this procedure was repeated three times. The ether solutions were now combined and washed four times with 200 cc. of 0.2 per cent sodium hydroxide, and these alkaline extracts were washed with 100 cc. of ether. The combined ether solutions contained 431 mg. (*Fraction B*). All the alkaline solutions except the first were combined. The hormones recovered from this solution weighed 1179 mg. (*Fraction A<sub>2</sub>*).

*Isolation of 17-Dihydroequilenin from Fraction A<sub>2</sub>*—This fraction (1158 mg.) and 1.32 gm. of picric acid were recrystallized together from 4 cc. of absolute alcohol. The mother liquor and 0.4 cc. of an ice-cold saturated solution of picric acid in absolute alcohol used for the washing of the picrate were taken up in 100 cc. of ether and extracted with dilute sodium carbonate solution until practically no color was removed. The ether contained 591 mg. (*Fraction C*). The brick-red, crystalline picrate was twice recrystallized from absolute alcohol. The purified product melted at about 170° with decomposition beginning at 90°. The picric acid was removed in the usual manner with sodium carbonate. On recrystallization of the ether-soluble material (423 mg.) from benzene-acetone (5:1) 313 mg. of dihydroequilenin were obtained. The substance crystallized from the solvent in long colorless needles which turned yellow on prolonged exposure to daylight. The crystals slowly lost solvent of crystallization on standing at room temperature. For analysis the compound was dried in a

high vacuum ( $10^{-4}$  mm.) at  $80^{\circ}$ . The dihydroequilenin melted at  $215\text{--}217^{\circ}$  (corrected) with decomposition. The melt did not turn red on prolonged heating (6).

*Analysis*— $\text{C}_{18}\text{H}_{20}\text{O}_2$ . Calculated. C 80.55, H 7.52  
Found. " 80.53, " 7.61  
" 80.47, " 7.52

*Rotation*— $[\alpha]_D^{25} = -5^{\circ}$  (0.7% in dioxane)

The absorption spectrum of a sample recrystallized three more times from the above solvent mixture is given in Fig. 1, Curve 2 (maxima at 231, 270, 280, 292, 328, and  $343\text{ m}\mu$ ).

*17-Dihydroequilenin Diacetate*—14.5 mg. of dihydroequilenin were dissolved in 2 cc. of acetic anhydride, refluxed for 45 minutes, and kept at room temperature for 24 hours. The excess of acetic anhydride was decomposed by the addition of ice-cold water; the diacetate was taken up in a benzene-ether mixture. This extract was washed with dilute sodium carbonate solution and water and yielded on evaporation 19.6 mg. of a yellow oil. The melting point ( $115\text{--}117^{\circ}$ , corrected) was constant after three recrystallizations from 80 per cent ethyl alcohol. 10 mg. of fine, colorless needles were obtained. Even the pure compound showed a marked tendency to separate as an oil on recrystallization.

*Analysis*—(Sample dried at  $80^{\circ}$ , 4 mm. Hg)

$\text{C}_{22}\text{H}_{24}\text{O}_4$ . Calculated, C 74.96, H 6.87; found, C 74.95, H 6.77

*17-Dihydroequilenin Di-p-Nitrobenzoate*—18 mg. of dihydroequilenin, 62 mg. of *p*-nitrobenzoyl chloride, and 1.4 cc. of dry pyridine were heated in a sealed tube on a steam bath for 4 hours and allowed to stand overnight. The ester was precipitated out by water, centrifuged, and washed with dilute ammonia and water. The crude product (25.7 mg.) was recrystallized three times from absolute alcohol-benzene (about 1:1). The yellow needles melted at  $251\text{--}252^{\circ}$  (corrected) with decomposition.

*Analysis*—(Sample dried in a high vacuum at  $80^{\circ}$ )

$\text{C}_{22}\text{H}_{24}\text{N}_2\text{O}_8$ . Calculated. C 67.82, H 4.63, N 4.95  
Found. " 67.45, " 4.88, " 5.10

*17-Dihydroequilenin-3-Monobenzoate*—126 mg. of dihydroequilenin were dissolved in 12.5 cc. of 5 per cent sodium hydroxide solution. An excess of benzoyl chloride was added in small

portions, with vigorous shaking after each addition until the precipitation of the benzoate was complete. The precipitate was washed with dilute sodium hydroxide solution and water. The crude product (152 mg.) was recrystallized twice from 90 per cent ethyl alcohol and once from absolute ethyl alcohol-benzene (5:1). The benzoate melted at 203–205° (corrected) with slight decomposition.

*Analysis*—(Sample dried at 80°, 0.06 mm. Hg)

$C_{28}H_{24}O_4$ . Calculated, C 80.60, H 6.50; found, C 80.32, H 6.60

*Oxidation of Dihydroequilenin Monobenzoate; Equilenin Benzoate*—59 mg. (0.32 milli-equivalent) of dihydroequilenin monobenzoate melting at 202° were dissolved in 9.5 cc. of glacial acetic acid (distilled over chromium trioxide) and 0.48 milli-equivalent of chromium trioxide in 90 per cent acetic acid was added. The mixture was kept at about 20° for 15 hours, then diluted with 70 cc. of ice-cold water. The precipitate was centrifuged and washed with 20 cc. of water. It was taken up in ether and extracted with 2 per cent sodium hydroxide and finally with water. The ether yielded 47.2 mg. This product was oily and apparently grossly contaminated with by-products. On five recrystallizations (three from absolute alcohol, two from 85 per cent acetone) 5.4 mg. were obtained melting at 223° (corrected), which is the melting point of Girard's equilenin benzoate. There was no depression of the melting point on mixing with an authentic sample.

*Analysis*—(Sample dried in a high vacuum at 80°)

$C_{28}H_{22}O_4$ . Calculated, C 81.04, H 5.99; found, C 80.28, H 6.16

*Isolation of  $\beta$ -Estradiol from Fraction B*—This fraction, which still contained red pigments, was distributed between 25 cc. of ether and 115 cc. of 2 per cent sodium hydroxide, and the extraction repeated seven times with 25 cc. of alkali. The combined alkaline extracts were twice washed with 40 cc. of benzene-petroleum ether (4:1). The alkaline phase was acidified and extracted with ether, which yielded 375 mg. of yellow crystals. A substance was present in this fraction exhibiting purple fluorescence in alkaline solution as well as in organic solvents. Since its absorption spectrum (maxima at 361 and 378  $m\mu$ ) extends into the range characteristic of dihydroequilenin (343  $m\mu$ ), it had to be removed

by recrystallization from benzene and acetone, and from 80 per cent alcohol. The absorption spectrum of the resulting crystals (177 mg.) showed the presence of 6 per cent of dihydroequilenin and about 12 per cent of Compound 3. The crystals were dissolved in 150 cc. of ether and extracted five times with 60 cc. portions of 0.2 per cent sodium hydroxide. The alkaline extracts were washed with 80 cc. of ether. The combined ether fractions yielded 145 mg. This material was recrystallized three times from the above solvents. 71 mg. of colorless needles were obtained melting at 222–224° (corrected) with decomposition. The melting point was not depressed by mixing with a sample of  $\beta$ -estradiol obtained by reduction of estrone.

*Analysis*—(Sample dried at 110°, 6 mm. Hg)

$C_{18}H_{24}O_2$ . Calculated, C 79.36, H 8.89; found, C 79.39, H 8.85

This preparation was free of dihydroequilenin but contained about 6 per cent of Compound 3. The removal of the latter contamination by recrystallization or by distribution between alkali and ether was found to be impracticable. Most of it could be eliminated, however, by selective adsorption under conditions similar to those used by Duschinsky and Lederer (28) for the isolation of ketonic estrogens. 67 mg. of material of this type—most of it derived from the mother liquors—were dissolved in 45 cc. of benzene, and the solution filtered slowly through a column of Brockmann's aluminum oxide (length 12.5 cm., diameter 6 mm.). An equal volume of benzene was used for developing. The more unsaturated compounds, dihydroequilenin and Compound 3, were retained in the upper part of the column, while  $\beta$ -estradiol accumulated below. Especially with less pure preparations the color reaction with *p*-nitrodiazobenzene gave valuable information about the distribution of the various diols. For this purpose a small amount of the adsorbent was extracted in a small test-tube by short contact with a few drops of alcohol, and the reagent added. The mixture was made alkaline with sodium hydroxide and then acidified with acetic acid. Dihydroequilenin gives a red color, while  $\beta$ -estradiol develops a yellow color on this treatment. Compound 3 is recognized by the development of a yellow color on the addition of the diazo reagent without alkali. Additional information is obtained by the fluorescence color on addition of concen-



trated sulfuric acid to the adsorbent. The elution was effected with 95 per cent alcohol in a Soxhlet apparatus. The resulting  $\beta$ -estradiol (48 mg.) was twice recrystallized and melted at 221–223.5° (corrected).

$$[\alpha]_D^{25} = +56^{\circ} \text{ (0.6\% in 95\% alcohol)}$$

The rotation of the synthetic product was reported as  $[\alpha]_D^{24} = +53.8^{\circ}$  (0.7 per cent in dioxane) (15), and  $[\alpha]_D^{18} = +56.7^{\circ}$  (in ethanol) (16). The absorption spectrum (Fig. 1, Curve 4) exhibits maxima at 281  $m\mu$  and 288  $m\mu$ . No measurements were taken in the far ultraviolet (12).

*$\beta$ -Estradiol-3-Monobenzoate*—12 mg. of  $\beta$ -estradiol were dissolved by warming with 4 cc. of 5 per cent sodium hydroxide solution and benzoylated in the same manner as dihydroequilenin. The crude benzoate (14.8 mg.) was recrystallized three times from 90 per cent ethyl alcohol. The melting point was sharp (151.5–152.5°, corrected) and constant, but was lower than that of the synthetic product (156–157.5°) (15). An explanation for this discrepancy was found in the fact that this benzoate occurs in three modifications. When the synthetic compound was heated beyond its melting point, the melt resolidified partly on cooling to –80°. The crystals thus obtained melted at +63°, but on further heating this melt resolidified between 105–125° and finally melted at 153°. If the heating was stopped as soon as the mass appeared to be completely molten, the melt resolidified on cooling (approximately 130°). The melting point of this product was 158°. This series of transformations is readily reproducible. The benzoate of the isolation product showed essentially the same behavior. If sulfuric acid instead of paraffin oil was used as the bath liquid for the melting point apparatus, the product gradually turned brown and the melting points of the various modifications were found to be lower. When the two preparations were mixed, there was no depression of the melting point (156.5°). Seeding with the synthetic product raised the melting point to 156°.

*Analysis*—(Sample dried at 110°, 6 mm. Hg)

$C_{28}H_{38}O_2$ . Calculated, C 79.74, H 7.50; found, C 79.66, H 7.35

*$\beta$ -Estradiol Diacetate*—11.5 mg. of  $\beta$ -estradiol were acetylated in the manner described for dihydroequilenin. The crude

diacetate (15.6 mg.) was recrystallized three times from 80 per cent alcohol. The final product (8.1 mg.) showed no depression of the melting point (140–142°, corrected) when mixed with a sample of synthetic  $\beta$ -estradiol diacetate.

*Analysis*—(Sample dried at 110°, 6 mm. Hg)

$C_{22}H_{28}O_4$ . Calculated, C 74.12, H 7.92; found, C 74.34, H 7.71

The spectrum exhibits maxima at 270 and 276  $m\mu$  and minima at 274 and 248 to 254  $m\mu$ . This is in accordance with our measurements on the diacetate of  $\alpha$ -estradiol obtained by reduction of estrone.

*Isolation of Compound 3 from Fraction C*—The fraction was recrystallized from 75 per cent alcohol, which removed the fluorescing substance also encountered in the purification of  $\beta$ -estradiol. The mother liquors of three subsequent recrystallizations were worked up in the manner described below. Suitable fractions were combined with the main fraction. The crystals were freed from dihydroequilenin by a series of distribution procedures similar to that used in the fractionation of non-ketonic phenols. The dihydroequilenin accumulated in the alkaline phase (0.4 per cent solution of sodium hydroxide, half saturated with sodium chloride). The addition of sodium chloride apparently effected a better separation. If the alkaline solution was fully saturated with sodium chloride (29), no dihydroequilenin was extracted; the material removed by the latter process exhibited an intense but rather uncharacteristic spectrum extending over the whole ultraviolet range. The final ether fraction was once recrystallized from ethyl acetate. 45 mg. were obtained which contained less than 3 per cent dihydroequilenin. This fraction was dissolved in 25 cc. of benzene-acetone (30:1), filtered through a 12.5 cm. aluminum oxide adsorption column (diameter 6 mm.), and washed with an equal volume of the same solvent mixture. The pigments still present were adsorbed in a narrow ring on the top of the column. 18 mg. were eluted with acetone from the zone underneath (7 cm.) and again adsorbed on aluminum oxide in the above manner. As the zone giving the yellow color with *p*-nitrodiazobenzene contains appreciable amounts of estradiol, the upper half of this zone was eluted separately. It yielded 6 mg. that were recrystallized from 80 per cent alcohol. The sub-

stance melted with decomposition at 220–223.5°, when the temperature was raised at a rate of 3° per minute.

*Analysis*—(Sample dried at 110°, 6 mm. Hg)

$C_{15}H_{22}O_2$ . Calculated, C 79.95, H 8.21; found, C 79.88, H 8.40

The color reactions obtained with the final preparations of the three compounds are given in Table II.

TABLE II  
*Color Reactions*

	Concentrated sulfuric acid		Coupling with <i>p</i> -nitrodiasobenzene		
	Color of solution	Color of fluorescence		After addition of alkali	On acidification with acetic acid
Dihydroequilenin . . .	Orange-yellow	Greenish yellow	Wine-red	Purple	Wine-red
$\beta$ -Estradiol . . . . .	Faint green	Blue	Faint yellow	"	Yellow
Compound 3 . . . . .	Greenish yellow	Green	Brown	"	Reddish brown

#### SUMMARY

Two estrogenic diols, 17-dihydroequilenin and  $\beta$ -estradiol, have been isolated from the urine of pregnant mares. Their physiological potencies are much less than those of the corresponding ketones. A third compound of the composition  $C_{15}H_{22}O_2$ , which is in all probability an unsaturated diol, has also been obtained in small amounts.

We wish to express our sincere thanks to Dr. E. Schwenk of the Schering Corporation of Bloomfield, New Jersey, for supplying us with the hormone concentrate and for providing means for the bioassays, to Dr. A. Girard of Paris who sent us samples of equilenin and its benzoate for comparison, to Mrs. M. Anchel for a preparation of carboxymethoxylamine, and to Mr. William Saschek for the microanalyses.

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## **THE EXCHANGE OF SALT AND WATER BETWEEN MUSCLE AND BLOOD**

### **IV. CORRECTION OF VALUES FOR VOLUME PHASES OF SKELETAL MUSCLE. METHODS FOR DETERMINATION OF BLOOD VOLUME IN MUSCLE**

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The purpose of this paper is to present data on the volume phases of skeletal muscle, both when the values are uncorrected and when they are corrected for the blood circulating within the muscle. A method is presented for the estimation of the amount of this circulating blood by the use of colloidal thorium dioxide, and the values obtained by this method are compared with those obtained from hemoglobin determinations.

In Paper I of this series (1), experiments were described in which the relative proportions of extra- and intracellular phases of skeletal muscle were estimated from determinations of the water and inorganic constituents of serum and muscle. From experimental data and the assumption that all of the muscle chloride is in the extracellular phase, it was possible to calculate the extracellular phase and thereby the intracellular phase per kilo of muscle. Since the chloride concentration per kilo of muscle indirectly represented the magnitude of the extracellular phase, it was evident that the volume phases could be estimated more accurately if correction were made for the chloride content of the blood circulating within the muscle. In order to make this correction, it was first necessary to determine the amount of blood so circulating. Heretofore, such estimations have been made from hemoglobin determinations. To substantiate these findings it was necessary to introduce into the blood stream a

foreign substance which would meet the following requirements: (1) It must remain at a constant level in the blood for a sufficient time for sampling; (2) it must not move freely across the barrier between blood plasma and the extracellular phase; and (3) it must not be stored in skeletal muscle.

In recent years many investigators have studied the storage of thorium in the various organs of the body after the intravenous injection of colloidal thorium dioxide. Most of the thorium dioxide (as much as 75 per cent) was stored in the spleen and liver; lesser amounts were found in the bone marrow and lung, traces in the kidney and adrenal, and none in the heart, muscle, brain, stomach, and large and small intestines (2-5). As in the case of bacteria, India ink, dyes, and other foreign substances injected intravenously, the particles of thorium dioxide are removed from the circulating blood by the reticulo-endothelial cells. Histological preparations as well as chemical analyses have established these findings (3). Therefore the conclusions reached by Sullivan, Neckermann, and Cannon (6) in the study of injected bacteria would appear to apply also to the thorium dioxide; that is, in the organs containing the most macrophages and a sinusoidal type of blood flow the primary localization occurs, whereas minimal localization occurs in organs poorly supplied with macrophages and with a rapid blood flow through vessels lined with ordinary endothelium.

The speed with which the reticulo-endothelial cells removed the particles of  $\text{ThO}_2$  from the blood stream had not been determined, although Leipert (2) reported that at the end of 24 hours the concentration of the stored thorium in the organs was the same as that found 26, 78, or 477 days following the injection. Therefore it appeared that all of the injected colloidal  $\text{ThO}_2$  had been stored within the 24 hours. The concentration of  $\text{ThO}_2$  in the blood stream immediately following the injection had not, however, been determined.

#### EXPERIMENTAL

Normal healthy dogs (used in all experiments) were anesthetized with nembutal, given intravenously in doses of 25 mg., dissolved in 0.5 cc. of water, per kilo of body weight. After the

dog was anesthetized for 15 minutes, a cannula was introduced into the femoral artery for the removal of samples of blood. The dogs then usually received an intravenous injection of approximately 0.8 cc. per kilo of body weight of thorotrast containing 0.25 gm. of  $\text{ThO}_2$  per cc. of solution. Samples of blood were taken under oil at varying intervals and the rectus abdominis muscle was removed at a designated time.

The following determinations were made on the serum: water, chloride, and thorium; on the blood: chloride, hemoglobin, cell volume, and thorium; on the muscle: water, chloride, hemoglobin, fat, and thorium.

### *Methods*

All muscle analyses were corrected for fat. The technique for the removal and treatment of blood and muscle was the same as described in Paper I (1). The chemical methods were also the same as those used in the preceding work, except that the hemoglobin of the muscle was determined by the acid hematin method of Whipple (7), and the red cell volume was determined with the Van Allen hematocrits (8).

*Determination of Thorium in Blood and Muscle*—To determine the thorium content of *serum* or *blood*, 1 cc. of serum or blood was placed in a  $20 \times 2.5$  cm. digestion tube, 2 cc. of concentrated  $\text{H}_2\text{SO}_4$  and 1.5 cc. of concentrated  $\text{HNO}_3$  were added, and the organic matter was destroyed by the usual digestion method over the free flame. After the liquid had become water-clear, the digestion was continued for 10 to 15 minutes to convert all the thorium into the sulfate. The tube was then cooled and placed in an ice bath. The contents were diluted with about 10 cc. of water, added at first drop by drop, so that the temperature of the solution would not rise above  $10^\circ$ , this hydrate of thorium sulfate being soluble at  $0^\circ$ . If a precipitate persisted after the addition of the water, the tubes were left in the ice box overnight, after which the solution was always clear. This solution was transferred quantitatively to a conical 50 cc. centrifuge tube, 1 drop of 0.1 per cent phenol red was added, and concentrated ammonium hydroxide was introduced slowly from a burette until the solution was alkaline. This procedure separated the thorium from the



calcium. The precipitated thorium hydroxide was collected by centrifugation. The supernatant solution was decanted and the precipitate washed with 10 cc. of water and again centrifuged. 2 drops of concentrated HCl were added to the washed and drained precipitate, the precipitate dissolving at once. The contents of the tube were then transferred with 10 cc. of water to a 15 cc. conical tube having a long slender point. 1 cc. of a 5 per cent oxalic acid solution was added; the contents were stirred and the tube was allowed to stand in the ice box overnight. The tube was then centrifuged, the supernatant liquid decanted, and the tube drained. A very fine solid glass rod, about  $3.5 \times 0.05$  cm., was placed in the tube before it was centrifuged to facilitate the drainage of the tube. The sides of the tube and the precipitate were washed three separate times with 5 cc. of water, and then centrifuged and drained. The washed thorium oxalate was dissolved by adding 2 cc. of 1:4  $\text{H}_2\text{SO}_4$  and placing the tube in a boiling water bath. The resultant solution was titrated with 0.01 N  $\text{KMnO}_4$ . 1 cc. of 0.01 N  $\text{KMnO}_4$  is equivalent to 0.66 mg. of  $\text{ThO}_2$ .

To determine thorium in *muscle*, 10 to 15 gm. of muscle, placed in a 50 cc. platinum dish, were dried overnight in a  $100^\circ$  oven and then ignited overnight in a muffle furnace at  $600^\circ$ . The contents of the dish were transferred quantitatively with water to a  $20 \times 2.5$  cm. test-tube. After the volume was concentrated to about 5 cc., 2 cc. of concentrated  $\text{H}_2\text{SO}_4$  and 1 gm. of anhydrous sodium sulfate were added and the contents digested over a microburner until sulfuric acid fumes filled the tube. The digest was boiled briskly for 15 minutes. The tube was cooled and placed in an ice bath. From this point on the procedure was the same as for serum.

*Note*—If between 1 and 2 mg. of thorium were used for each analysis, a more accurate determination resulted. If larger amounts of thorium were present, the contents of the tubes of dissolved thorium sulfate were transferred quantitatively to volumetric flasks and an aliquot equivalent to 1 to 2 mg. of thorium was used for each analysis. The data obtained from analyses of serum and muscle to which known amounts of colloidal thorium dioxide had been added showed a recovery of 99 per cent in muscle and 100 per cent in serum.

### Results

Eleven experiments were conducted on normal anesthetized dogs following the intravenous injection of colloidal thorium dioxide. Representative data are given in Table I. Those experiments not included did not differ essentially from those reported.

*Concentration of Thorium Dioxide in Blood Stream*—Although the amounts of  $\text{ThO}_2$  injected were approximately the same, the concentration in the blood stream assumed different levels in different dogs. However, in all dogs there was a decrease in the concentration of thorium in the blood stream 1 to 3 minutes after the injection, followed by a period (about 30 minutes) during which the concentration remained fairly constant. These results are shown in Table I and presented graphically in Fig. 1. During the period of constant concentration of  $\text{ThO}_2$ , there was no change of quantitative significance in the chloride concentration or water content of the serum, red cells, or muscle; the red cell volume also remained constant.

*Concentration of Thorium Dioxide in Muscle*—The muscle was always removed within the period that the thorium concentration was fairly constant in the blood stream (Table I, Fig. 1). The concentration of thorium dioxide per kilo of fat-free muscle was small, varying from 0.311 mm to 0.500 mm. Representative data are given in Column 9.

*Estimation of Blood Volume in Skeletal Muscle*—Since colloidal thorium dioxide is removed from the blood stream by the reticulo-endothelial system, and skeletal muscle is so poorly supplied with macrophages, only minimal amounts of thorium dioxide should be stored in muscle. This conclusion was substantiated histologically by Angermann and Overhof (3), and confirmed by chemical analyses as follows: An 8 kilo dog was given an intravenous injection of 6 cc. of thorotrast. 44 hours after the injection, blood and the rectus abdominis muscle were removed simultaneously. No thorium was found in either the blood or the muscle. Therefore, the amount of thorium dioxide in the skeletal muscle at the time of removal should depend upon the amount of blood circulating within that muscle, provided that the thorium particles did not move across the barrier between blood plasma

TABLE I

*Serum, Blood, and Muscle Analyses after Intravenous Injection of Colloidal ThO<sub>2</sub>*

Injected solution (thorotrast), 25 per cent colloidal ThO<sub>2</sub>. Muscle values corrected for neutral fat.

Time after injection (1)	[H <sub>2</sub> O] <sub>s</sub> (2)	[Cl] <sub>s</sub> (3)	[Th] <sub>s</sub> (4)	[Cl] <sub>b</sub> (5)	Hematocrit (6)	[H <sub>2</sub> O] <sub>M</sub> (7)	[Cl] <sub>M</sub> (8)	[Th] <sub>M</sub> (9)	Blood in muscle		(F) <sub>1</sub> (12)	(F) <sub>2</sub> (13)
									ThO <sub>2</sub> method (10)	Hb method (11)		
Dog VII-T; weight 13 kilos; 10 cc. thorotrast injected; no urine												
min.	gm. per gm.	mM per l.	mM per l.	mM per l.		gm. per gm.	mM per kg.	mM per kg.	cc. per kg.	cc. per kg.	gm. per kg.	gm. per kg.
3			10.65		42							
7	0.9160		10.30									
11		109.2										
17	0.9166		10.30	89.4		0.7638	23.10	0.480	80	80	190	132
25		109.2	10.30	89.0	42							
31	0.9181	111.2	10.30	90.5								
Dog VIII-T; weight 12 kilos; 10 cc. thorotrast injected; no urine												
3	0.9293		12.6		47							
6			13.0									
10	0.9290	110.1	12.7	86.8	47							
18			12.3		48	0.7691	21.60	0.390	64	72	179	133
25	0.9316	110.1	11.4	85.6	48							
28			11.4		48							
Dog IX-T; weight 13 kilos; 11 cc. thorotrast injected; 50 cc. urine, containing no thorium												
3	0.9299		9.00									
6		107.8	8.30	86.4	43							
10	0.9300	108.0	9.01		44	0.7836	23.80	0.311	61	64	200	156
18			9.50	86.3	43							
25	0.9307	108.5	9.00	86.8								
Dog X-T; weight 14 kilos; 11 cc. thorotrast injected; no urine												
5			10.80	83.1								
8	0.9202	106.1	9.30	83.1	51							
11			8.37			0.7698	22.60	0.329	81	82	192	135
20	0.9202		8.42	83.1	52							
25			8.40	84.1	52							

and the extracellular phase. That such movement did not occur was demonstrated as follows: A 10 kilo dog was given an intravenous injection of 9 cc. of thorotrast, after which 100 cc. of isotonic glucose solution were injected intraperitoneally. After 45 minutes the peritoneal fluid was removed. Although the chloride concentration of the glucose solution within the peritoneum had risen to 87 mm per liter, there was no thorium dioxide present.

From the concentration of  $\text{ThO}_2$  in the blood and muscle, the volume of blood per kilo of fat-free muscle was estimated and is

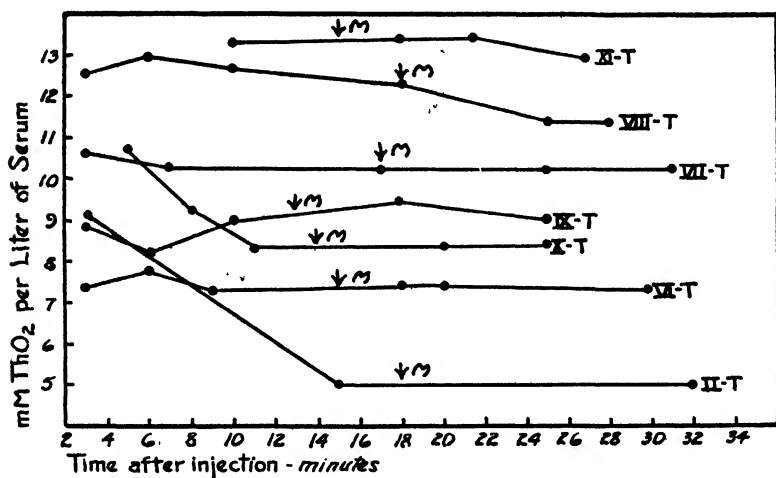


FIG. 1. Concentration of thorium dioxide in the blood following intravenous injection of thorotrast. Each experiment is represented by a line; the arrows indicate the time of removal of the rectus abdominis muscle (M). The numerals represent dog numbers.

given in Column 10 of Table I. A comparison of this blood volume was made with that estimated from hemoglobin concentrations (Column 11). The latter was only slightly higher than the volume estimated from thorium determinations. This discrepancy may be due to the extraction of some of the myoglobin from the muscle or to the slight turbidity that occurred in some of the acid hematin solutions of the muscle extracts, or to both.

*Correction of Volume Phases of Muscle*—From the volume of blood per kilo of fat-free muscle, it was possible to estimate the concentration of blood chloride in that muscle, and to correct

the total chloride of muscle for the chloride of the blood circulating therein. To show the effect of this correction on the estimation of the volume phases of muscle the analytical data have been subjected to the same mathematical treatment as that described in detail in Paper I of this series.<sup>1</sup>

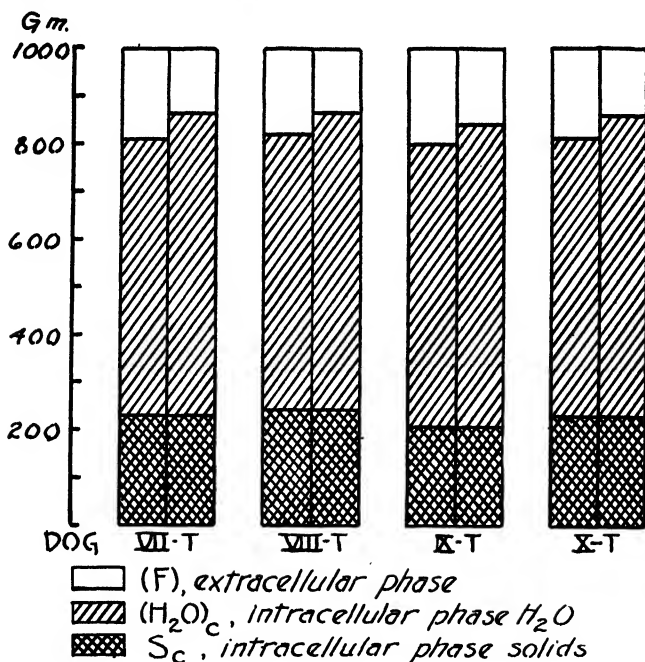


FIG. 2. Intracellular water and solids and extracellular phase of 1 kilo of muscle. The first column of each pair presents the data for fat-free muscle, uncorrected for the blood circulating within it; the second column presents the corrected data for blood-free muscle.

The uncorrected amount of extracellular phase (F)<sub>1</sub> in gm. per kilo of muscle was calculated from the equation

$$(F)_1 = \frac{(\text{Cl})_M \times (\text{H}_2\text{O})_s \times 1000}{1.04 \times (\text{Cl})_s}$$

<sup>1</sup> In this discussion, the subscripts 1 and 2 will be used to denote volume phases uncorrected and corrected, respectively, for the blood circulating in the muscle.

in which the subscripts  $M$  and  $s$  represent muscle and serum respectively. These values are given in Column 12 of Table I.

The corrected amount of extracellular phase  $(F)_2$  in gm. per kilo of muscle was calculated from the equation

$$(F)_2 = \frac{(Cl)_M - (Cl)_{bM} \times (H_2O)_s \times 1000}{1.04 \times (Cl)_s}$$

in which the subscript  $bM$  represents the blood circulating within the kilo of muscle.

On the basis of these values for  $(F)_1$  and  $(F)_2$  the extra- and intracellular phases of 1 kilo of muscle have been calculated:  $(F)_1 = 181, \sigma 12$ ;  $(F)_2 = 136, \sigma 10$ ; intracellular phase  $(C)_1 = 819, \sigma 12$ ;  $(C)_2 = 864, \sigma 10$ ; percentage of water in intracellular phase  $\{H_2O\}_{C_1} = 71.6$ ;  $\{H_2O\}_{C_2} = 73.0$ .

The estimated changes are graphically presented in Fig. 2.

*Conclusion*—Normal fat-free skeletal muscle of dogs, exclusive of the blood circulating therein, contained a maximum of 16 mm of chloride per kilo. This value was found to be remarkably constant, and as a result the extracellular phase volume was quite constant, but lower than the volume values reported in previous papers, in which the calculations were not based on blood-free muscle.

#### SUMMARY

1. A method is presented for the determination of thorium in muscle and blood.

2. Colloidal thorium dioxide was found not to move freely across the barrier between blood plasma and extracellular phase, and was not stored in skeletal muscle.

3. In excised skeletal muscle of dogs, the volumes of blood present were estimated from thorium and hemoglobin determinations, and found to agree within limits of experimental error.

4. Experiments on normal dogs are described in which the volume of extra- and intracellular phases of skeletal muscle have been estimated from blood-free muscle. From the corrected data the conclusions reached were (a) the extracellular phase amounts to a maximum of 14 per cent, and the intracellular phase to 86 per cent, of the muscle; (b) the percentage of water in the intracellular phase is 73.0.

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## STUDIES ON THE MERCAPTURIC ACID SYNTHESIS IN ANIMALS

### VIII. *L*-CYSTINE, *DL*-METHIONINE, GLUTATHIONE, AND TAURINE IN RELATION TO THE SYNTHESIS OF MERCAPTURIC ACIDS IN THE RAT

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Bromobenzene (1) and naphthalene (2) check the growth of rats which are maintained on a diet low in organic sulfur but adequate for moderate growth. Growth is resumed when *L*-cystine or *DL*-methionine, but not when taurine, is incorporated in the bromobenzene or the naphthalene diet. As additional evidence for the participation of *L*-cystine and *DL*-methionine in the detoxication of bromobenzene and naphthalene in the rat, a study of the extent of the synthesis of mercapturic acids in relation to dietary *L*-cystine and *DL*-methionine appeared desirable. It will be recalled that it has been demonstrated that these two sulfur-containing amino acids augment the synthesis of *p*-bromophenyl-mercapturic acid in the dog (3).

Because it has been suggested that glutathione is involved in the detoxication of bromobenzene in the animal body (4), and because of the conclusion of Dyer and du Vigneaud (5) that glutathione may yield cystine or cysteine during metabolism, the rôle of glutathione in the synthesis of mercapturic acids was also investigated.

#### EXPERIMENTAL

Male albino rats, 4 months old, were used. The animals were kept in individual metabolism cages which were suspended over large funnels containing porcelain filters to separate the feces from the urine. The urine of each rat was collected daily, and



the combined excretion for a 3 day period was sampled for analysis. The urine was preserved with toluene.

The composition of the diets which were used in the present study is described in Table I. In order to obtain comparable results, the food intake of each animal was kept constant throughout the investigation. Each rat was fed a weighed portion of food daily. 400 mg. of yeast powder and 100 mg. of cod liver oil were given separately. The rats which at any time during the experiment showed reluctance to consume the weighed amount of food within 24 hours were discarded, and the results obtained were deleted. The diets which were low in sulfur and contained

TABLE I  
Composition of Diets\*

Diet No.....	C-0	C-6	C-10	C-16
	gm.	gm.	gm.	gm.
Casein†.....	0	6	10	16
Sucrose.....	15	15	15	15
Corn-starch.....	56	50	46	40
Salt mixture‡.....	4	4	4	4
Lard.....	25	25	25	25

\* Each rat received daily 400 mg. of dried yeast (Northwestern Yeast Company) and 100 mg. of cod liver oil in addition to the basal diet.

† Casein 453, Casein Company of America, Inc.

‡ Osborne and Mendel salt mixture (Osborne, T. B., and Mendel, L.B., *J. Biol. Chem.*, **37**, 572 (1919)).

a comparatively large amount of naphthalene (1 per cent) or bromobenzene (1 or 2 per cent) were, as a rule, the most troublesome in this respect. Only fourteen out of twenty-seven of these animals consumed the entire amount of food within the desired period. Water was allowed *ad libitum* to all animals.

The rats were fed the basal diets which contained bromobenzene or naphthalene in amounts varying from 0.5 to 2.0 and from 0.25 to 1.0 per cent respectively, and the excretion of the corresponding mercapturic acids in the urine was estimated. To each of the diets were then added either 500 mg. of *l*-cystine, 550 mg. of *dl*-methionine, 500 mg. of taurine, or 1.28 gm. of glutathione per 100 gm. of the diet. The amount of food offered was that consumed by the same rat when no supplementary sulfur compounds were present in the diet.

The urine collected over a 3 day period was made up to 100 cc. with distilled water, and the mercapturic acids were determined in duplicate by Stekol's method (6). For comparative studies such as described here, the method seems sufficiently accurate,<sup>1</sup> but by it, however, one cannot differentiate the mercapturic acids from any other similar substance which on alkaline hydrolysis might yield an aromatic mercaptan. In experiments in which any such compound was suspected, in addition to or in lieu of the mercapturic acid, isolation of the mercapturic acid was made by a procedure which was essentially that of McGuinn and Sherwin (7).

The bromobenzene was redistilled and the naphthalene was resublimed before use. *l*-Cystine, *dl*-methionine, glutathione, and taurine were commercial products. *p*-Bromophenylcysteine was prepared from *p*-bromophenylmercapturic acid by the method of Baumann and Schmitz (8). All of the substances were analytically pure.

### Results

*Synthesis of l- $\alpha$ -Naphthalenemercapturic Acid*—For the sake of economy of space the results on only one rat are shown in Table II. Similar data were obtained with the other five animals. Rats which consumed 82.5 mg. of naphthalene in 3 days (0.25 per cent of naphthalene in the diet) synthesized about the same amount of mercapturic acid whether they received practically no protein or 16 per cent of protein in the diet. Neither *l*-cystine nor methionine augmented the synthesis of mercapturic acid under these conditions. The rats, however, lost weight rapidly when no protein, cystine, or methionine was fed together with naphthalene. It seems that about 50 per cent of the 82.5 mg. of naphthalene consumed was all that the rat could convert into the mercapturic acid, and the cysteine which was necessary for the synthesis was derived from the tissues. Increasing the dose of naphthalene 100 per cent (165 mg. in 3 days) did not augment the synthesis of the mercapturic acid on the protein-free diet.

<sup>1</sup> The applicability of the method to rat urine was checked in recovery experiments with each of the mercapturic acids. The per cent recovery of the acids was as satisfactory as that found for dog urine (6). Lack of space does not permit presentation of the data.

The addition of *l*-cystine or methionine to the diet increased the synthesis by about 10 to 12 per cent. Taurine and glutathione were ineffective in this respect. On a 16 per cent casein diet the same dose of naphthalene (165 mg. in 3 days) yielded about the same amount of mercapturic acid as that obtained on a protein-

TABLE II  
*Extent of Synthesis of l-α-Naphthalenemercapturic Acid in Rat*

Experiment No.	Naphthalene (33 gm. food intake, 3 days)	Diet No.	Supplement	Mercapturic acid excreted	
				Total	As naphthalene detoxicated
	mg.			mg.	per cent
1	82.5	C-0	None	83.2	44.6
2	82.5	C-10	"	95.4	51.3
3	82.5	C-16	"	90.5	48.5
4	82.5	"	<i>l</i> -Cystine	94.4	50.3
5	82.5	"	Methionine	93.0	49.8
6	165.0	C-0	None	96.8	25.8
7	165.0	"	<i>l</i> -Cystine	106.2	28.4
8	165.0	"	Methionine	106.1	28.4
9	165.0	"	Glutathione	90.0	23.6
10	165.0	C-16	None	118.4	31.7
11	165.0	"	<i>l</i> -Cystine	155.0	41.5
12	165.0	"	Methionine	160.0	42.9
13	165.0	"	Glutathione	120.0	32.2
14	165.0	"	Taurine	118.0	31.6
15	330.0	"	None	155.0	20.8
16	330.0	"	<i>l</i> -Cystine	200.0	26.8
17	330.0	"	Methionine	215.0	28.8
18	330.0	"	Glutathione	151.3	20.2
19	330.0	"	Taurine	150.0	20.1

The data shown in this table represent experiments with one rat. Similar results were obtained with five other animals. The amounts of the supplements fed are mentioned in the experimental section of the text. 4 day rest periods were allowed between experiments. Purina Dog Chow was fed *ad libitum* during these intervals.

free diet supplemented with cystine or methionine. *l*-Cystine and methionine, but not glutathione or taurine, augmented the synthesis still further. The results obtained on feeding the 1 per cent naphthalene diet are similar in their general aspects to those obtained with the 0.5 per cent naphthalene diet (165

mg. in 3 days). Again, glutathione, like taurine, did not have any effect on the output of *l*- $\alpha$ -naphthalenemercapturic acid in the urine, whereas *l*-cystine and methionine were effective.

Inspection of the data in Table II shows that the capacity of the rat to synthesize *l*- $\alpha$ -naphthalenemercapturic acid is definitely limited, and that the extent of the synthesis of the mercapturic acid undoubtedly depends on the organic sulfur and the naph-

TABLE III  
*Extent of Synthesis of p-Bromophenylmercapturic Acid in Rat*

Experiment No.	Bromobenzene (26 gm. food intake, 3 days)	Supplement to Diet C-6	Mercapturic acid excreted	
			Total	As bromoben- zene detoxicated
	mg.		mg.	per cent
1	130	None	72.2	27.4
2	130	<i>l</i> -Cystine	105.8	40.2
3	130	Methionine	107.2	40.7
4	130	Taurine	73.2	27.8
5	130	Glutathione	74.4	28.3
6	260	None	97.2	18.5
7	260	<i>l</i> -Cystine	161.5	30.6
8	260	Methionine	173.9	33.1
9	260	Glutathione	93.0	17.7
10	260	Taurine	96.0	18.3
11	520	<i>l</i> -Cystine	274.8	26.1
12	520	Methionine	280.0	26.7
13	520	None	42.8	4.1
14	520	Glutathione	46.0	4.3

The data shown in this table represent experiments with one rat. Similar results were obtained with seven other animals. The amounts of the supplements fed are mentioned in the experimental section of the text. 4 day rest periods were allowed between experiments. Purina Dog Chow was fed *ad libitum* during these intervals.

thalene content of the diet. It will also be observed that the efficiency of the rat in converting the naphthalene into the mercapturic acid is greater the smaller the dose of naphthalene fed. This observation is similar to that reported with bromobenzene detoxication in dogs (9) and in rats (Table III). It seems probable that in the rat maintained on an adequate diet, as in the dog (9), the extent of the synthesis of mercapturic acids

is related to the body weight of the animal, not being necessarily identical for the two species.

Combined urines of rats which had been fed naphthalene and methionine with the protein-free diet contained 406 mg. of *l*- $\alpha$ -naphthalenemercapturic acid as estimated by analysis. Of this amount, 252 mg. of the mercapturic acid were isolated from the urine, and the analysis of the compound is shown below.<sup>2</sup>

	C	H	N	M.p. °C.
Found.	62.40	5.50	4.89	171
Calculated.	62.28	5.18	4.84	171

Of the 300 mg. of *l*- $\alpha$ -naphthalenemercapturic acid (as determined by analysis of the pooled urine) which were present in the urine of rats which had been fed naphthalene and glutathione with the protein-free diet, 193 mg. of the acid were isolated and identified by analysis.<sup>2</sup>

	C	H	N	M.p. °C.
Found.	62.33	5.40	4.74	171
Calculated.	62.28	5.18	4.84	171

The isolation of the acid from the urine does not, however, exclude the possibility that some other compound similar to the mercapturic acid (a derivative of homocysteine or glutathione, for example) was also present in the urine. The comparatively large amounts of the mercapturic acid isolated make it seem probable, however, that the mercapturic acid is the principal, if not the sole, substance of this type which is synthesized under these conditions.

<sup>2</sup> Upon addition of concentrated HCl to the naphthalene urine, in amounts approximately equal to one-tenth of the volume of the urine, and after letting it stand for several hours at room temperature, a purplish precipitate separated. From this precipitate we were able to isolate naphthalene (2) as well as some mercapturic acid. The larger portion of the mercapturic acid remained in the urine and was then extracted with chloroform. So far we have been unable to identify conclusively the nature of the rest of the purplish precipitate. In this connection the reported isolation of  $\alpha$ -naphtholglucuronic acid and of  $\alpha$ -naphtholpenturonic acid hydrate from the urine of rabbits which were fed naphthalene seems suggestive (10).

*Synthesis of p-Bromophenylmercapturic Acid*—Data shown in Table III were obtained on one rat. The results secured with seven other animals were similar and for the sake of brevity the data were omitted.

*l*-Cystine and *dl*-methionine, but not taurine or glutathione, greatly augmented the synthesis of *p*-bromophenylmercapturic acid in the rat. The amino acids were effective at all levels of bromobenzene administration (0.5, 1, or 2 per cent of the diet).

On the 2 per cent bromobenzene diet the rats rapidly lost weight, appeared depressed, and were sensitive to handling. It seems that 2 per cent of bromobenzene in the diet greatly exceeds the amount which the rat can detoxicate. Lough and Lewis (11) reported a similar observation made on the rabbit. With large doses of bromobenzene considerably less mercapturic acid (as measured by the rise in the neutral sulfur output in the urine) was excreted than that obtained with smaller amounts of bromobenzene. Our observations are in accord with those of Lough and Lewis (11), and offer no support for the contention of Haley and Samuelsen (12) regarding the non-toxicity of bromobenzene in the rat.

*Experiments with p-Bromophenylcysteine*—Brand and Harris (4) have suggested that as a possibility "one may conceive that the substances, which are being detoxified, first combine with glutathione, thereby rendering it more unstable, as a result of which the detoxified compound splits off in combination with part of the original glutathione molecule." The authors have further suggested that "*p*-bromophenylmercapturic acid referred to above contains an acetyl group, which may be a remnant of the glutamic acid radical of glutathione, rather than the result of acetylation." The failure of glutathione to augment the synthesis of mercapturic acids indicated that the tripeptide is not involved in the detoxication of either bromobenzene or naphthalene. As an additional test of the above theory, we administered *p*-bromophenylcysteine in doses varying from 155 to 630 mg. to four different rats. The compound was fed mixed with the food for 3 to 4 days and during the period the urine was collected in the manner described above. *p*-Bromophenylmercapturic acid was isolated from the urine of each rat. A typical analysis of the compound follows.

	C	H	Br	N	M.p. °C.
Found.	41.77	3.91	25.16	4.43	151
Calculated.	41.54	3.77	25.16	4.40	151-152

The data demonstrate that *p*-bromophenylcysteine can be directly acetylated in the organism of the rat to yield *p*-bromophenylmercapturic acid.

#### DISCUSSION

In considering the mechanism of the alleviation of the "deficiency imposed by bromobenzene and naphthalene" in the rat maintained on a low casein diet, at least two factors must be taken into account. One is the replacement by either *l*-cystine or methionine of that cysteine which is lost by the rat as a mercapturic acid. The other factor is the increased output of mercapturic acids which is induced by the presence of the supplements in the diet. Our data show that under the conditions of the experiments in the rat, as in the dog (3), *l*-cystine and methionine contribute to both factors mentioned above; and the promotion of growth of rats on bromobenzene and naphthalene diets low in casein (1, 2) by cystine and methionine may be due to replacement of the lost cysteine or to increased synthesis of mercapturic acid or to both, depending on the amount of bromobenzene or naphthalene consumed by the animal.

We suggested previously (2, 3) that bromobenzene and naphthalene when fed to animals attack the tissue to yield the corresponding mercapturic acids, and that the dietary organic sulfur, when available, is utilized by the animal to repair the attacked tissue. In the light of the above suggestion, our observation that glutathione, unlike *l*-cystine and *dl*-methionine, does not augment the synthesis of mercapturic acids would appear to indicate that the tripeptide is not utilized by the rat to form new tissue. Our results on the synthesis of mercapturic acids offer no evidence that cystine or cysteine is liberated from glutathione during metabolism in the rat. Furthermore, the data indicate that glutathione is apparently not involved in the synthesis of either of the mercapturic acids in the manner suggested by Brand and Harris (4).

Although it may seem possible that the ingestion of naphthalene and bromobenzene modified the normal metabolism of

glutathione in the rat, preliminary experiments (13) showed that glutathione promotes the growth of rats which are fed naphthalene with the low casein diet. In this respect the metabolic behavior of glutathione in the rat under the conditions of our experiments seems to be similar to that on low casein diets devoid of naphthalene or bromobenzene (5).

Additional experiments on glutathione and other sulfur-containing compounds in relation to growth and the synthesis of mercapturic acids in animals are at present in progress. Pending their completion we will postpone a fuller discussion of the implications of our results with glutathione.

The author wishes to express his gratitude to Mr. J. Alicino for the microanalytical work.

#### SUMMARY

1. *L*-Cystine and *dl*-methionine, but not glutathione or taurine, augmented the synthesis of *l*- $\alpha$ -naphthalenemercapturic and *p*-bromophenylmercapturic acids in rats maintained on low sulfur diets.

2. The efficiency of the rat in converting naphthalene or bromobenzene into the corresponding mercapturic acids is greater the smaller the dose of naphthalene or bromobenzene fed.

3. The experiments support our previous suggestion that the dietary sulfur determines the extent of the synthesis of mercapturic acids only in so far as it affects the nutritional state of the animal.

4. *l*- $\alpha$ -Naphthalenemercapturic acid was isolated from the urine of rats which were fed glutathione or *dl*-methionine with a protein-free diet containing naphthalene.

5. The synthesis of *p*-bromophenylmercapturic acid from *p*-bromophenylcysteine in the rat has been established.

6. The data show that the cysteine and the acetyl group of the mercapturic acid are not derived necessarily from glutathione in the manner suggested by Brand and Harris (4).

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# THE PARTITION OF URINARY NITROGEN OF FASTING AND HIBERNATING WOODCHUCKS (*ARCTOMYS MONAX*)

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During the past few years systematic studies have been made in this Laboratory of the metabolism of the woodchuck during periods of fasting and hibernation. In connection with these studies, collections of the urine have been made from time to time and the nitrogen partition has been determined in these urines. The animals were confined in metal and glass cages and the urines were collected in receptacles in which was placed 10 per cent hydrochloric acid in amounts that were estimated sufficient to keep the urine continually acid as it was spontaneously voided by the animals. The urines were collected and measured and preserved and the analyses made subsequently. The determinations were made by the method of Folin and associates (1).

Valentin (2) determined only the percentage of urea by weight in the urines of hibernating animals. The only previous systematic study of the urines of marmots during hibernation was made by Nagai (3) who used the method of Pfaundler (4) for separating the nitrogen into fractions by means of phosphotungstic acid. He found a shift in the so called "ammonia" fraction in that in hibernation it was about half the value found during fasting and that the "amino acid" fraction was markedly increased during hibernation as compared with that found during fasting or when the animal was on food.

The results obtained with one animal in the Nutrition Laboratory series are given in Table I. The animal was deprived of food on November 15, 1932, and the urines were collected at intervals during the period of fasting until December 3, at which time the animal was again given food. Food was given inter-

## 344 Partition of Urinary N of Woodchucks

TABLE I

*Elimination and Distribution of Urinary Nitrogen in Fasting and Hibernating Woodchuck (Arctomys monax)*

Period* and condition	Body weight	Total N		Distribution of N in per cent of total N					Preformed creatinine	Creatine as creatinine	Creatinine† coefficient
		Per 24 hrs.	Per kilo per 24 hrs.	Amino acid N	Ammonia N	Urea N	Preformed creatinine N	Creatine N			
1838	kg.	mg.	mg.						mg.	mg.	
Fasting											
Nov. 15-17	1.70	614	361	1.1	12.2	79.0	1.6	0.0	53.9	1.47	16
" 17-19	1.61	504	313	0.6	4.2	86.3	1.8	1.2	48.6	33.2	15
" 19-21	(1.54)	373	242	0.5	4.2	85.1	2.4	0.0	47.4	0.69	15
" 21-23	1.48	316	214	0.9	4.1	84.6	2.8	1.6	47.6	26.5	16
" 23-28	1.40	260	186	0.5	4.7	82.2	2.6	2.5	91.3	87.3	13
" 28-Dec. 3	1.24	264	213	0.6	5.1	85.9	2.4	4.0	84.5	141	14
On food											
Dec. 3- 5	(1.25)	266	213	0.9	7.7	82.0	2.3	2.8	32.3	40.2	13
" 5- 7	1.28	866	677	0.8	11.4	81.9	1.5	0.7	70.7	34.5	28
" 7- 9	1.28	637	498	0.6	7.1	87.7	1.1	0.4	39.3	14.1	15
" 9-12	1.25	836	669	0.9	6.2	84.8	1.1	0.3	71.1	19.0	19
Fasting											
Dec. 12-14	1.30	684	526	0.8	5.5	83.6	1.1	1.0	39.7	36.8	15
" 14-17	1.23	375	307	0.5	4.9	85.1	1.5	1.1	43.9	32.8	12
" 17-20	1.13	336	297	0.6	5.2	80.0	1.7	3.3	45.6	88.1	13
" 20-31‡	1.13	32.9	29.1	0.7	5.2	80.9	1.9	0.3	18.8	2.46	1.5
1833											
Dec. 31-Jan. 7	1.05	62.1	59.1	0.6	4.6	76.0	1.9	0.2	22.2	2.59	3.0
Jan. 7-14‡	1.02	37.1	36.4	1.0	4.8	89.0	2.1	0.4	14.4	2.94	2.0
" 14-16	(1.02)	111	109	1.0	4.8	85.6	1.7	0.6	10.3	3.71	5.0
" 16-21	1.02	49.8	48.8	1.0	4.8	81.3	1.8	0.5	12.3	3.44	2.4
" 21-30‡	0.99	60.3	60.9	1.0	4.5	74.5	1.6	0.4	23.2	6.29	2.6
" 30-Feb. 4‡	0.96	49.0	51.0	1.0	4.8	88.1	1.8	0.3	11.7	1.66	2.4
Feb. 4- 6	0.94	201	214	0.6	4.7	80.8	1.3	0.2	14.1	1.76	7.5
" 6-15	0.91	85.2	93.6	0.8	4.1	76.6	0.9	0.4	19.3	8.22	2.4
" 15-24	0.91	29.2	32.1	1.0	4.3	84.3	1.8	1.0	12.5	7.06	1.5

The values included in parentheses are estimated.

\* All periods began and ended at 9 a.m.

† Preformed. The coefficient is given in terms of mg. per kilo per 24 hours.

‡ In hibernation.

mittently in the period December 3 to 12, at which time the long fast began. From December 12 until February 24 the animal was fasting and during certain periods hibernated. These periods were December 20 to 31, January 7 to 14, January 21 to 30, and January 30 to February 4. The animal died on February 27. The animal was kept in a cold environment during the entire period of time.

The nitrogen per kilo per 24 hours varied widely and in the period before the long fast began ranged from 186 to 677 mg. During the long fast it dropped rapidly until the minimum of 29.1 mg. per kilo was reached in the first period of hibernation, December 20 to 31. All periods of hibernation gave low values. The period, January 14 to 16, as well as February 4 to 6, had a value somewhat higher than the general level during fasting.

The percentage of nitrogen as amino acids ranged from 0.5 to 1.1. Although the percentage averaged higher in the latter portion of the long fast, it is questionable whether there is any significance to the difference between the general level during the fast and that preceding the long fast.

The percentage of nitrogen as ammonia, for the most part, was under 5. The per cent as ammonia may be used as an indication of the preservation of the urine. From this standpoint it would appear that in the periods November 15 to 17 and December 5 to 7 the urines had not been adequately preserved, but certainly during the long fast the values were extremely uniform and there is no reason to believe that the urines were not adequately preserved.

The percentage of nitrogen as urea ranged extremely high, considering the fact that the animal was fasting, that it was herbivorous, and that there was hibernation accompanied by extremely wide variation in the nitrogen level of the animal. Although allantoin determinations were not carried out, it would seem as though the amount must have been small, as the nitrogenous materials other than urea contained such a relatively small percentage of the total nitrogen that was eliminated.

The percentage of nitrogen as preformed creatinine ranged from 0.9 to 2.8. In the period before the long fast began the percentage as creatinine was slightly higher than during the long fasting period.

During most of the periods of feeding and fasting there was some creatine elimination and in some cases there was actually a larger amount of creatine than of creatinine. The per cent of nitrogen as creatine ranged from 0 to 4.0 in the entire series.

The creatinine coefficient is of interest as an indication of the condition of nutrition of the animal as to whether it was fat or lean. Also the uniformity of the creatinine coefficient from period to period may be used as an indication of the completeness of collection of the urine. From the first period, November 15 to 17, through to the third period of the long fast, December 17 to 20, the creatinine coefficient varied between 12 and 28 mg. per kilo per 24 hours. The value of 28 mg. per kilo is an exceptional one and no explanation has been found for it. With this exception, the values did not have a wide range considering the conditions under which the urines were collected. The effect of hibernation, however, was shown very markedly in the sharp drop in this value in the period of December 20 to 31. From there on, with the exception of the period February 4 to 6, the values were all below 5 mg. per kilo. It will be noted that the coefficient of 7.5 accompanies the high nitrogen per kilo of 214 mg.

A striking finding in all the series of determinations is the lack of any marked change in the percentage of nitrogen of the various constituents. In spite of a tremendous fall in the nitrogen elimination either per day or per kilo per 24 hours, there was no significant change in the per cent of total nitrogen as amino acids, ammonia, urea, and preformed creatinine. The hibernation of woodchucks is accompanied by a very marked fall in the total metabolism. Therefore the protein metabolism follows in general the same direction quantitatively as the total metabolism and qualitatively there appears to be no change in the protein metabolism.

The findings presented are typical of those with other animals, the results of which, as well as a complete discussion of the various phases of the physiology and metabolism of these animals under normal waking conditions and in hibernation, will be found in a forthcoming monograph of the Carnegie Institution of Washington by Dr. Francis G. Benedict and Mr. Robert C. Lee.

Acknowledgment is made to Dr. Francis G. Benedict for the opportunity to make these analyses through the inauguration of the general study of the physiology of hibernation.

## SUMMARY

The urine of a woodchuck (*Arctomys monax*) was collected during periods of fasting, with food, and hibernation, and the total nitrogen, amino acids, ammonia, urea, creatine, and creatinine were determined. In spite of extreme variations in the level of nitrogen elimination, the percentage distribution of nitrogen in the urine was unaffected by either prolonged fasting or by hibernation. Quantitatively there was an extraordinary fall in the protein metabolism especially in hibernation, but qualitatively the protein metabolism was not altered.

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# THE RÔLE OF THE ACETYL DERIVATIVE AS AN INTERMEDIARY STAGE IN THE BIOLOGICAL SYNTHESIS OF AMINO ACIDS FROM KETO ACIDS\*

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There was a twofold purpose in the undertaking of the present investigation. First we wished to demonstrate, if possible, the actual conversion of one optical isomer into another within the animal body by direct proof, and, secondly, we wished to throw light on the mechanism of the *in vivo* synthesis of amino acids from the corresponding keto acids.

If the conversion of one isomer into another in the body could be demonstrated, substantial ground would be afforded for the hypothesis which has been offered to explain how the unnatural enantiomorph can support growth of animals in lieu of the natural isomer in the case of certain amino acids, such as tryptophane (1, 2), methionine (3), homocystine (4), and histidine (5). As previously discussed by Berg and Potgieter (1) and by du Vigneaud, Sealock, and Van Etten (2) in connection with the utilization of *d*-tryptophane, the unnatural isomer is probably oxidatively deaminized to the non-asymmetric keto acid and from this the naturally occurring isomer is asymmetrically synthesized.

In the present series of experiments we have been able to demonstrate by direct proof a biological conversion of one optical isomer into the other. After the feeding of *l*-phenylaminobutyric acid to the dog, we have been able to isolate from the urine the acetyl derivative of the *d*-phenylaminobutyric acid. That the body can convert one isomer into its enantiomorph is also indicated by the

\* A preliminary report of this work was presented before the meeting of the American Society of Biological Chemists at Detroit, April 10-13, 1935.



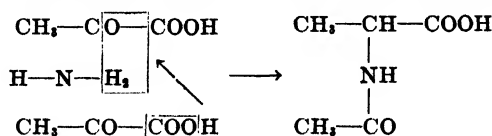
work of Conrad and Berg (6) which has just recently been reported. These investigators were able to demonstrate that more *l*-histidine was laid down in the tissues of animals on an *l*-histidine-deficient diet supplemented with *d*-histidine than could be attributed to the small amounts of *l*-histidine in the diet.

In addition to demonstrating an actual conversion of isomers, the experiments reported here also serve to support the hypothesis originally proposed by Knoop for the synthesis of amino acids in the animal body. This hypothesis which involves the acetyl derivative as an intermediate step was based on the following considerations.

In 1910 Knoop (7) in studying the deamination of amino acids in intermediary metabolism decided to use the next higher homologue of phenylalanine, phenylaminobutyric acid. He felt that with the homologue the reaction would be qualitatively the same as for the naturally occurring amino acid but that the rate of the reactions might be slowed up. Consequently, there would be more chance of intermediary compounds being excreted in the urine. *dl*-Phenylaminobutyric acid was, therefore, fed to dogs and the excretory products were studied (8). Some of the amino acid was isolated from the urine, but a greater proportion of the levorotatory component than of the dextro was found to be present. Small amounts of *d*-phenylhydroxybutyric acid and some hippuric acid were also obtained. In addition, a substance was isolated which proved to be the acetyl derivative of the *d*-phenylaminobutyric acid. This curious observation led to further investigations. The corresponding keto acid was fed and here, likewise, the same acetyl derivative was obtained as after feeding of the *dl*-amino acid. The acetyl-*dl*-phenylaminobutyric acid was then fed (9). However, in this instance, an excess of the acetyl-*l*-phenylaminobutyric acid was obtained in the urine. This showed that the body could actually oxidize the acetyl-*d*-better than the acetyl-*l*-phenylaminobutyric acid and the conclusion was drawn that the dextrorotatory amino acid was probably the isomer corresponding in spatial configuration to the naturally occurring series of amino acids, the truth of which we have been able to demonstrate by the physicochemical method of Lutz and Jirgensons and which we will discuss later in this paper.

It was quite clear from these experiments that the *dl*-amino acid could not have been directly acetylated and then selectively oxidized, for, otherwise, the acetyl-*l* isomer should have appeared in the urine and not the acetyl-*d* acid. As pointed out by Knoop and Blanco, the isolation of the acetyl-*d*-phenylaminobutyric acid after feeding of the keto acid was no doubt due to an asymmetric synthesis of the acetyl-*d*-phenylaminobutyric acid from the keto acid, and the excretion of this same acetyl derivative from the feeding of the *dl*-amino acid may have resulted from the oxidative deamination of the amino acid to the keto acid, followed by asymmetric synthesis to the acetyl-*d*-phenylaminobutyric acid. Parallel studies were made with phenylaminoacetic acid and results identical with those from the higher homologue were obtained. Neubauer and Warburg (10) had also obtained evidence of acetylation of amino acids from perfusion studies. After the perfusion of the liver with *dl*-phenylaminoacetic acid they isolated an optically active acetylphenylaminoacetic acid which was identical with that isolated later by Knoop and Blanco from the urine of the dog.

From the results he had obtained Knoop advanced at that time the hypothesis that acetylation was an actual intermediary step in the production of an amino acid from the corresponding keto acid. He suggested that the reaction might be accomplished through the condensation of the keto acid with ammonia and pyruvic acid in a manner similar to the *in vitro* reactions studied by Erlenmeyer and Kunlin (11, 12) and by de Jong (13). These workers had shown that, when a keto acid is heated with a solution of ammonium carbonate, apparently 1 molecule of the keto acid unites with a molecule of ammonia and is reduced at the cost of another molecule of the keto acid, which in turn is oxidized to the next lower fatty acid, and is left attached to the amino group so that an acylamino acid results. In some instances the reactions have proceeded almost quantitatively. Thus, when pyruvic acid is heated with ammonium carbonate, acetylalanine is formed in a high yield, the reaction being represented by de Jong as follows:



In similar fashion Erlenmeyer (14) showed that glyoxylic acid, ammonia, and pyruvic acid yielded acetylglycine. It is a very significant fact that Knoop was able to increase the yield of acetylamino acid excreted in the urine when pyruvic acid was fed simultaneously with the phenylketobutyric acid. On the other hand, butyric or acetic acid did not augment the amount of acetylphenylaminobutyric acid excreted.

In a continuation of these studies Knoop and Blanco (9) turned to phenylalanine and fed the acetyl derivative of the racemic form, expecting the body to oxidize the acetyl-*l*-phenylalanine and to excrete the acetyl-*d*-phenylalanine. Contrary to their expectations, they obtained from the urine a levorotatory acetylphenylalanine,  $[\alpha]_D = -50.3^\circ$ , which from various experiments they were led to believe was the acetyl derivative of the naturally occurring *l*-phenylalanine. This interpretation of the data led in turn to the conclusion that the body could burn more readily the acetyl derivative of the unnatural isomer. Knoop and Blanco (9), therefore, abandoned the acetyl theory as a normal step in metabolism, stating, after discussing the results obtained with phenylalanine:

"Das beweist u. E. besonders deutlich, dass für diese Aminosäure ein Acetylierungsprozess als normaler Vorgang nicht in Betracht kommt. Diese Vorstellung ist also für den vorliegenden Fall zu verlassen.—Es müssen andere Gründe für die Acetylierung auch der 2 Homologen vorliegen und wir vermuten, dass dieselbe einfach das Ergebnis einer Reaktionsfähigkeit einer intermediären Abbauphase mit anderen Stoffwechselzwischenprodukten darstellt, ohne dass darin ein besonderer Nutzen für den Tierkörper zu liegen braucht."

The belief that the keto acid is an intermediary step in the catabolism of amino acids and that the keto acid can be converted to the amino acid in the body was not abandoned by Knoop since the experimental results with phenylalanine did not affect these aspects of the theory. In fact, further work by Knoop and co-workers has emphasized the metabolic importance of keto acids and imino acids. In *in vitro* model experiments Knoop and Oesterlin (15) have shown that imino acids are very readily reduced by hydrogen in the presence of a catalyst such as platinum and have suggested that various oxidizable substances, including pyruvic acid, might serve in place of hydrogen as the reducing agents *in*

*vivo*. It is, therefore, clear that the phenylalanine results caused them to abandon only the pyruvic acid-acetylation theory of synthesis as the normal step in the metabolic synthesis of amino acids. As we expect to show, however, the interpretation of the phenylalanine results was based on a misconception.

In some racemization studies on amino acids, du Vigneaud and Meyer (16) had occasion to resolve phenylalanine and prepared the *d*- and *l*-phenylalanines and their acetyl derivatives. The acetyl derivative of *d*-phenylalanine turned out to have the same rotation,  $[\alpha]_D^{20} = -51^\circ$ , as the acetyl derivative isolated from urine by Knoop and Blanco and considered by them to be the acetyl derivative of the naturally occurring *l*-phenylalanine. It was, therefore, suspected that those investigators had, in reality, isolated acetyl-*d*-phenylalanine and that contrary to their conclusion the body could oxidize more readily the acetyl derivative of the naturally occurring levo isomer.

It can readily be appreciated that the ingenious theory which Knoop had built up on the basis of his work with phenylamino-butyric acid and phenylaminoacetic acid, if true, would be extremely important to our theories of amino acid synthesis in the body. Because of the great importance to the theory of the identity of the isomer of phenylalanine excreted, we felt that the actual physiological work of Knoop and Blanco should be repeated, and that we should determine directly the isomer which was more easily oxidized by the body.

In the preparation of the compounds for these physiological studies we again resolved phenylalanine and prepared the acetyl derivatives of the enantiomorphs. The observations of du Vigneaud and Meyer with respect to the configurational relationship between the isomers of phenylalanine and their acetyl derivatives were confirmed.

The physiological experiments of Knoop and Blanco were then repeated and we have been able to confirm their actual experimental data; that is, that after the feeding of acetyl-*dl*-phenylalanine a levorotatory acetyl derivative is excreted in the urine. However, this acetyl derivative is actually that of the unnatural isomer. In other words, the body is able to burn more readily the acetyl derivative of the naturally occurring form, and therefore in our opinion the basis upon which Knoop's theory of the

acetyl derivative as a normal intermediate in the synthesis of amino acids was abandoned no longer exists.

If the original explanations set forth by Knoop for the experimental observations he obtained with *dl*-phenylaminobutyric acid be correct, a crucial experiment becomes apparent, as foreshadowed by statements of Knoop. It should follow that the feeding of *l*-phenylaminobutyric acid should result in the excretion of the acetyl derivative of *d*-phenylaminobutyric acid. This prediction is based on the conception that the *d*-phenylaminobutyric acid corresponds in spatial configuration to the natural series of amino acids. Accordingly, the *l*-phenylaminobutyric acid should be oxidatively deaminized to the keto acid, and from the keto acid the acetyl derivative of the dextrorotatory phenylaminobutyric acid would be asymmetrically synthesized. On this basis, if enough of the *l*-phenylaminobutyric acid were fed, some of the acetyl-*d*-phenylaminobutyric acid would be expected to be excreted in the urine.

For these studies the phenylaminobutyric acid was prepared by a modification of Fischer's method of synthesis (17) which we worked out. The resolution was accomplished through the brucine salt of the formyl derivative and both optical isomers were isolated. In contrast to phenylalanine, the acetyl derivatives had the same direction of rotation as the hydrochlorides of the corresponding free amino acids.

After feeding *dl*-phenylaminobutyric acid to two dogs we were able to isolate acetyl-*d*-phenylaminobutyric acid from the urines in yields comparable to those of Knoop. The *l*-phenylaminobutyric acid was then fed to a number of dogs and according to prediction the acetyl derivative of the *d*-phenylaminobutyric acid was isolated from the urine in each case. It was identical in crystalline form, melting point, and rotation with the synthetic compound. Considering the difficulty in isolation and the consequent amounts lost, the isolation accounted for a large proportion of the amount of the *l* acid fed. In addition, small amounts of the hydroxy acid and hippuric acid were obtained from the urine.

In order to make a final check on our general interpretation of the explanation of the conversion of the isomers, we also fed the *d* isomer. We wished to rule out the possibility that contrary to

what we expected the *d*-phenylaminobutyric acid might yield the acetyl-*l*-phenylaminobutyric acid. However, after the feeding of the *d* isomer, acetyl-*d*-phenylaminobutyric acid was isolated from the urine. Also of interest was the fact that the amount of acetyl-*d*-phenylaminobutyric acid obtained was about the same after the feeding of each isomer. This would be expected if the theory were correct, since both enantiomorphs should be deaminized to the keto acid. Although some oxidation does take place as witnessed by the isolation of hippuric acid, the oxidizability of the keto acid is apparently poor. Some is converted to hydroxy acid, but the greater proportion is converted to the acetyl derivative which accumulates and is excreted. Any hydrolysis of the latter which might occur would simply liberate the free acid. This would then go through the cycle once more. That the body can hydrolyze the acetyl-*d*-phenylaminobutyric acid is indicated by the fact that the feeding of the acetyl-*dl*-phenylaminobutyric acid is followed by the excretion of an excess of the acetyl-*l*-phenylaminobutyric acid.

It is readily seen that the significance of the data obtained with phenylaminobutyric acid to the metabolism of ordinary amino acids depends in great measure upon the assumption that the dextrorotatory phenylaminobutyric acid corresponds in spatial configuration to the naturally occurring series. From the results of studies with the isomers of the acetyl derivatives of the other amino acids it is justifiable to assume that the acetyl derivative less readily handled by the body corresponds in configuration to the unnatural series. For example, although the body can utilize for growth purposes both *d*- and *l*-tryptophane (1, 2), only the acetyl-*l*-tryptophane can be utilized (2, 18). The acetyl-*d*-tryptophane will not support growth of animals on a tryptophane-deficient diet. Likewise both enantiomorphs of methionine (3) and homocystine (4, 19) have been demonstrated to be utilizable for growth in connection with the proper experimental conditions, but of the acetyl or formyl derivatives, only the derivative of the naturally occurring isomer can be utilized. The same relationship has been shown to be true of oxidizability; the acetyl derivatives of *d*-cystine (20) and of *d*-homocystine (19) are far less completely oxidized than the free amino acid, whereas acetylation of the isomer corresponding to the natural series causes practically no

difference in the oxidizability. No exception to these relationships has so far been found. However, to place this beyond any doubt, it was felt desirable to test this conclusion with regard to the spatial configuration of *d*-phenylaminobutyric acid by the method of Lutz and Jirgensons (21). These workers have been able to show that all the amino acids of the naturally occurring

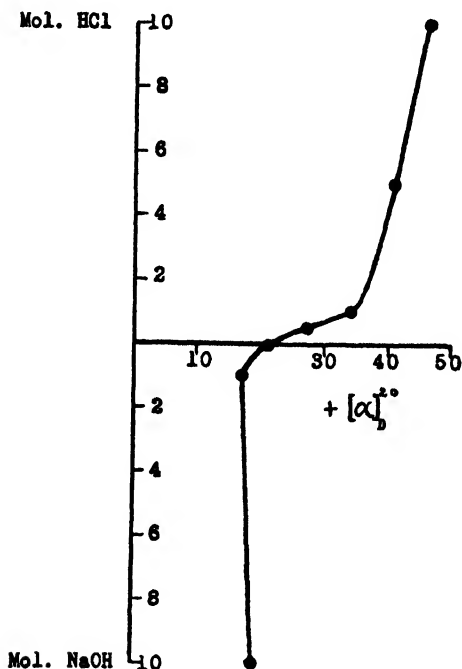


FIG. 1. Variation in the specific rotation of *d*-phenylaminobutyric acid in 0.0067 M concentration with different molecular equivalents of HCl and NaOH.

series show a change in the rotation towards the more negative side with decreasing amounts of HCl. The specific rotation of the *d*-phenylaminobutyric acid was, therefore, determined with varying molecular equivalents of HCl and NaOH and, as shown in Fig. 1, the characteristic drift to the negative side was found. The *d*-phenylaminobutyric acid is, therefore, without question the isomer corresponding in spatial configuration to the naturally

occurring amino acids and, according to recent suggestions (22), ought to be designated the *l*-(+)-phenylaminobutyric acid. We have retained in this paper the older terminology used by Knoop so as not to cause confusion in the understanding of the relationship in the results of the two papers. We would suggest, however, that in the future the newer designation be used.

#### EXPERIMENTAL

*Preparation of  $\gamma$ -Phenyl- $\alpha$ -Aminobutyric Acid*—A mixture of 122 gm. of phenylethyl alcohol and 500 gm. of hydrobromic acid. (23) was refluxed for 6 hours. The oily upper layer was washed successively with concentrated  $H_2SO_4$ , water, and finally with 10 per cent  $(NH_4)_2CO_3$ . The product was distilled *in vacuo*. The yield of phenylethyl bromide was 170 gm., which is 92 per cent of the theoretical amount.

To a solution of 85 gm. of sodium in 1500 cc. of absolute alcohol were added 750 gm. of diethyl malonate followed by 585 gm. of phenylethyl bromide. After the mixture had been refluxed for 5 hours the alcohol was removed *in vacuo* and the residue was washed several times with water. The oily layer was distilled *in vacuo* and the fraction boiling at 132–134° under 3 to 4 mm. pressure was collected. 700 gm. of the phenylethylmalonic ester were obtained.

445 gm. of the above ester were added slowly to 265 gm. of KOH in 1325 cc. of 50 per cent alcohol and the mixture was refluxed gently for 1 hour. The alcohol was removed completely by vacuum distillation. During the distillation water was added from time to time so that the final volume was about 1 liter. The solution was then cooled in an ice bath and was acidified with an excess of HCl. During the addition of the HCl care was taken to keep the temperature of the reaction mixture below 20°. The phenylethylmalonic acid was then extracted with 1500 cc. of alcohol-free ether in four portions. The combined ether extracts were dried with  $MgSO_4$  and were treated in an ice bath with 215 gm. of dry bromine. The brominated ether solution of the acid was added to 3 liters of concentrated  $NH_4OH$ . The mixture was allowed to stand for 2 days, whereupon the excess  $NH_3$  was removed *in vacuo*. 420 cc. of concentrated HCl were added and the mixture was refluxed for 1 hour. The  $\gamma$ -phenyl- $\alpha$ -aminobutyric



acid was precipitated by neutralization with  $\text{NH}_4\text{OH}$ . The crude product was purified by dissolving it in 1500 cc. of hot water which contained enough  $\text{HCl}$  to effect solution, filtering the solution through norit, and precipitating the compound with  $\text{NH}_4\text{OH}$ . The product was filtered and washed first with water and then with alcohol. Recrystallization by the above procedure was repeated until the  $\gamma$ -phenyl- $\alpha$ -aminobutyric acid obtained had a melting point of  $305\text{--}306^\circ$  (corrected). 180 gm. of pure product were obtained which represented a yield of 60 per cent of the theoretical amount, based on the phenylethylmalonic ester used. The product had the following composition.

$\text{C}_{10}\text{H}_{13}\text{O}_2\text{N}$ .	Calculated.	C 67.02, H 7.31, N 7.82
	Found.	" 66.85, " 7.59, " 7.84

*Preparation of dl- $\gamma$ -Phenyl- $\alpha$ -Formylaminobutyric Acid*—71.5 gm. of pure *dl*- $\gamma$ -phenyl- $\alpha$ -aminobutyric acid dissolved in 860 cc. of redistilled formic acid were treated gradually with 260 cc. of pure acetic anhydride. The temperature was held at  $70\text{--}75^\circ$  for 30 minutes after the addition of the acetic anhydride had been completed. 600 cc. of water were then added and the solution concentrated *in vacuo* with the addition of water from time to time, until the acetic acid had been removed. The residue was dissolved in the minimum amount of methyl acetate and crystallized by the addition of petroleum ether. 79 gm. of *dl*- $\gamma$ -phenyl- $\alpha$ -formylaminobutyric acid melting at  $130\text{--}131^\circ$  (corrected) were obtained. This is 95 per cent of the theoretical amount. The compound gave the following analysis.

$\text{C}_{11}\text{H}_{13}\text{O}_3\text{N}$ .	Calculated, N 6.76; found, N 6.84
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*Resolution of dl- $\gamma$ -Phenyl- $\alpha$ -Formylaminobutyric Acid and Isolation of the Isomers of the Free Amino Acid*—57 gm. of the above formyl derivative were intimately mixed in a mortar with 109 gm. of brucine. The mixture was then dissolved in a minimum amount of boiling anhydrous methyl alcohol. After the solution was refluxed for 15 minutes, the brucine salt began to crystallize. The solution was allowed to cool and an equal volume of methyl acetate was added. The mixture was allowed to stand in the ice box for 48 hours and the product was filtered. The precipitate amounted to 55 gm. and had a rotation of  $[\alpha]_D^{20} = -19.0^\circ$  for a

1 per cent solution in methyl alcohol. The filtrate was concentrated to one-fifth its volume and was allowed to stand in the ice box overnight. A second crop of crystals amounting to 10.2 gm., with a rotation of  $[\alpha]_D^{30} = -23.0^\circ$ , was obtained. Both fractions melted at  $160-162^\circ$  (corrected). Recrystallization of the salt from boiling absolute methyl alcohol gave a product with a rotation of  $[\alpha]_D^{30} = -23.2^\circ$  and a melting point of  $160-162^\circ$  (corrected). To a cold solution of 44 gm. of this brucine salt in 1 liter of distilled water were added 65 cc. of cold concentrated  $\text{NH}_4\text{OH}$ . After the mixture had stood for 10 to 15 minutes in an ice bath, the brucine was filtered and washed with ice water. The filtrate was extracted with ten 60 cc. portions of chloroform followed by two extractions with ether. The solution was aerated for 1 hour to remove any ether and excess  $\text{NH}_3$ . After neutralization to litmus with  $\text{HCl}$ , sufficient concentrated  $\text{HCl}$  was now added to make the solution 1 N. The mixture was heated in a boiling water bath for 2 hours and then made faintly alkaline to litmus with  $\text{NH}_4\text{OH}$ . Crystallization of the *l*- $\gamma$ -phenyl- $\alpha$ -aminobutyric acid began promptly. The yield was 9.2 gm., with a rotation of  $[\alpha]_D^{30} = -47.0^\circ$  for a 1 per cent solution in 1 N  $\text{HCl}$ , and a melting point of  $323-325^\circ$  (corrected). Recrystallization from water did not change the rotation. The compound gave the following analysis.

$\text{C}_{16}\text{H}_{19}\text{O}_2\text{N}$ . Calculated, N 7.82; found, N 7.71

The original mother liquors from the crystalline brucine salt of *l*- $\gamma$ -phenyl- $\alpha$ -formylaminobutyric acid were concentrated *in vacuo* to a heavy syrup. An equal volume of methyl acetate was added and the mixture allowed to stand for 48 hours in the ice box. A very small amount of the brucine salt of the *l* acid precipitated and was filtered. To the filtrate, 500 cc. of water were added, the solution was cooled to  $0^\circ$ , and then was decomposed as described above for the preparation of *l*- $\gamma$ -phenyl- $\alpha$ -aminobutyric acid. 10 gm. of the *d*-phenylaminobutyric acid were thus obtained possessing a rotation of  $[\alpha]_D^{30} = +48.8^\circ$  for a 1 per cent solution in 1 N  $\text{HCl}$ . The product melted at  $326-328^\circ$  (corrected) and gave the following analysis.

$\text{C}_{16}\text{H}_{19}\text{O}_2\text{N}$ . Calculated, N 7.82; found, N 7.74

*Preparation of Acetyl-d-γ-Phenyl-α-Aminobutyric Acid*—5.0 gm. of *d*-γ-phenyl-α-aminobutyric acid were dissolved in 20 cc. of water and 3.86 cc. of 7 N NaOH. 3.21 cc. of 7 N NaOH and then 1.1 cc. of acetic anhydride were added to the solution cooled in ice. Eight such additions of NaOH and acetic anhydride were made at 2 minute intervals, the solution being shaken vigorously in the ice bath during the addition of the acetic anhydride. Especial care was taken to keep the solution alkaline to phenolphthalein at all times during the reaction in the presence of the acetic anhydride to prevent any racemization (24, 16). The reaction mixture was allowed to stand for 20 minutes at room temperature after the last addition of the reagents. 40.2 cc. of 5.14 N H<sub>2</sub>SO<sub>4</sub>, equivalent to the total amount of NaOH used, were then added.

The crystalline acetyl-*d*-γ-phenyl-α-aminobutyric acid was obtained by chilling the solution in an ice bath and filtering. The crude product was recrystallized once from absolute ethyl alcohol and finally from the minimum amount of hot water. The yield of pure substance, dried over calcium chloride, was 5.0 gm. A 1 per cent solution in absolute ethyl alcohol possessed a rotation of  $[\alpha]_D^{20} = +26.7^\circ$ . The compound melted at 179–180° (corrected) and gave the following analysis.

C<sub>13</sub>H<sub>15</sub>O<sub>4</sub>N. Calculated, N 6.33; found, N 6.31

*Preparation of Isomers of Acetylphenylalanine*—The acetyl-*dl*-phenylalanine was prepared by the acetylation of *dl*-phenylalanine with acetic anhydride in an alkaline aqueous solution. The compound possessed the properties previously described (9). The resolution was carried out according to the directions of du Vigneaud and Meyer (16), with the brucine salt of the formyl derivative. The active acetyl isomers were prepared by acetylation of the corresponding active phenylalanines (16). The acetyl derivative prepared from *d*-phenylalanine had a rotation of  $[\alpha]_D^{20} = -50.9^\circ$  in a 1 per cent absolute ethyl alcohol solution and melted at 172° (corrected), while that from the *l*-phenylalanine possessed a rotation of  $[\alpha]_D^{20} = +51.4^\circ$  and melted at 172° (corrected). To check the configurational identity beyond any question the acetyl derivatives were hydrolyzed and in each case the original isomer having the same rotation was obtained.

*Metabolic Experiments*

To test which acetyl derivative was handled better by the body and to ascertain the identity of the one excreted in the urine 10 gm. of the *dl*-acetylphenylalanine were administered to a dog which had been fasted for 24 hours. The compound was fed thoroughly mixed with ground meat over a period of 3 days and the urine collected during the period of feeding and for 5 days thereafter. The animal was kept in a metabolism cage with a false bottom to prevent fecal contamination of the urine. In addition, the collections of urine were made at frequent intervals and the feces washed from the cage whenever soiling was noted. The samples of urine were preserved under toluene. The urine from the first 4 days (700 cc.) was extracted separately from that of the second 4 day period. The latter volume was also about 700 cc.

Each lot was extracted with ether for 24 hours in a continuous extraction apparatus. At the end of the extraction period the ether extract was evaporated and the residue taken up in a small volume of water. This aqueous solution was rendered alkaline to litmus with NaOH and washed several times with ether and butyl alcohol. The solution was then made acid to litmus with HCl.

The solution was colored considerably and relatively large amounts of charcoal had to be used for clarification; no doubt, much of the compound desired was lost on the charcoal. The filtrate after decolorization was cooled in an ice bath and made acid to Congo red. The amount of acetylphenylalanine was in close agreement with that obtained by Knoop and the direction of rotation confirmed that reported by him. However, the results demonstrated that a larger amount of the acetyl derivative of the dextro isomer than of the *l* form is excreted, since the acetyl-*d*-phenylalanine has a negative rotation. From the first batch of urine 1.5 gm. of the analytically pure acetyl derivative were obtained, which possessed a rotation of  $[\alpha]_D^{25} = -3.7^\circ$  in a 1 per cent absolute ethyl alcohol solution. From the second batch of urine 1.0 gm. was obtained with a rotation of  $[\alpha]_D^{25} = -32.2^\circ$ . Thus from 10 gm. of acetyl-*dl*-phenylalanine 2.5 gm. of analytically pure acetylphenylalanine were actually isolated, representing a fairly good recovery of material considering the difficulties in isolation. Control experiments with acetylphenylalanine added to dog urine gave a recovery of only about 40 per cent of the

compound. Knoop reported a recovery of 4.68 gm. from 18 gm. fed to the dog. He reported that one fraction had a rotation of  $[\alpha]_D = -4.48^\circ$ , while another had a rotation of  $[\alpha]_D = -27.9^\circ$ . The results of these two series of investigations check one another remarkably well.

When the optically active acetyl-*d*-phenylalanine with a rotation of  $[\alpha]_D^{20} = -50.9^\circ$  was fed, the same isomer could be recovered in pure form from the urine. This result confirmed the actual data which Knoop obtained in his experiment with the acetyl derivative of phenylalanine of rotation  $[\alpha]_D = -50.3^\circ$ .

In all instances the acetyl derivatives isolated gave the correct analytical values for acetylphenylalanine.

For the experiments with acetyl-*dl*-phenylaminobutyric acid the compound was administered as described for phenylalanine. However, the animals did not seem to tolerate well the phenylaminobutyric acid and after the 1st day of feeding the animals had to be coaxed to partake of the food and in some instances they refused to eat on the 3rd day of the administration. Animals differed markedly in the amount which they would tolerate.

In a typical experiment, 16 gm. of the *dl*-phenylaminobutyric acid were fed and 2.05 gm. of acetyl-*d*-phenylaminobutyric acid were obtained, in confirmation of the results of Knoop. The compound melted at  $179-180^\circ$  (corrected) and possessed a rotation of  $[\alpha]_D^{23} = +28.0^\circ$  for a 1 per cent solution in absolute ethyl alcohol. The nitrogen value of 6.31 per cent agreed with the theoretical value of 6.33. A mixture of the derivative isolated from the urine and the synthetic acetyl-*d*-phenylaminobutyric acid showed no depression in melting point. The derivative was isolated from the urine in the manner described for the phenylalanine experiments.

The *l*-phenylaminobutyric acid was then fed to three animals and in each instance acetyl-*d*-phenylaminobutyric acid was recovered from the urine. For example, 12 gm. were fed to a dog under the conditions described previously and by methods already outlined 2.4 gm. of acetyl-*d*-phenylaminobutyric acid were isolated from the urine. In addition, 0.5 gm. of hippuric acid was obtained.

Finally one experiment with *d*-phenylaminobutyric acid was carried out. After the feeding of 20 gm. of *d*-phenylaminobutyric

acid we were able to isolate from the urine 3.5 gm. of acetyl-*d*-phenylaminobutyric acid.

A different method of extraction was resorted to for this last experiment. Much difficulty is encountered in the extraction of the urine with ether owing to emulsions forming in the extractor, and we have, therefore, tried to improve the method of extraction. Other acids were tried for acidification, but HCl seemed to be the most satisfactory. Evaporation of the urine to dryness, extraction of the residue with alcohol, evaporation of the alcohol, and extraction of the aqueous solution of the latter residue in a continuous extractor worked better than the extraction of the original urine. We finally worked out a method which gave somewhat better results. The new method is less time-consuming, less troublesome, and has given us slightly better recoveries of acetyl-*d*-phenylaminobutyric acid added to dog urine. About a 50 per cent recovery has been obtained. It was used in the feeding experiment with the *d* isomer. The urine sample is extracted with several portions of ethyl acetate. In the experiment with the *d* acid 1900 cc. of urine collected during 3 days after the feeding of the phenylaminobutyric acid were repeatedly extracted with ethyl acetate in a large separatory funnel. After each extraction the ethyl acetate layer was added to a distilling flask and the ethyl acetate distilled off under reduced pressure at a temperature of 38–50°. In this way, the ethyl acetate recovered could be used for fresh extractions. About ten extractions of the urine were made with 300 cc. portions of ethyl acetate. The residue resulting from the distillation of the ethyl acetate was recrystallized from the minimum amount of hot water, yielding 4.1 gm. of a product which upon recrystallization yielded 2.9 gm. of acetyl-*d*-phenylaminobutyric acid possessing a rotation of  $[\alpha]_D^{25} = +28.8^\circ$  for a 1 per cent solution in absolute ethyl alcohol and a melting point of 178–179° (corrected). From reworking the various mother liquors 0.3 gm. of similar product was obtained. From the urine of the second 3 day period a further amount of 0.3 gm. was obtained, yielding a total of 3.5 gm. of acetyl-*d*-phenylaminobutyric acid. A mixture of the isolated product with the synthetic acetyl-*d*-phenylaminobutyric acid showed no lowering of the melting point. This was true in all instances in which the acetyl-*d*-phenylaminobutyric acid had been isolated from the urine.

## DISCUSSION

We feel that the results reported in this paper place the pyruvic acid-acetylation theory for the *in vivo* synthesis of amino acids back in the position it so rightly deserves. Whether this theory is extensible to all the amino acids or whether it is confined to certain of the aromatic amino acids must await further research. Where the acetyl mechanism is involved, the metabolism of the amino acid might be represented as in Fig. 2.

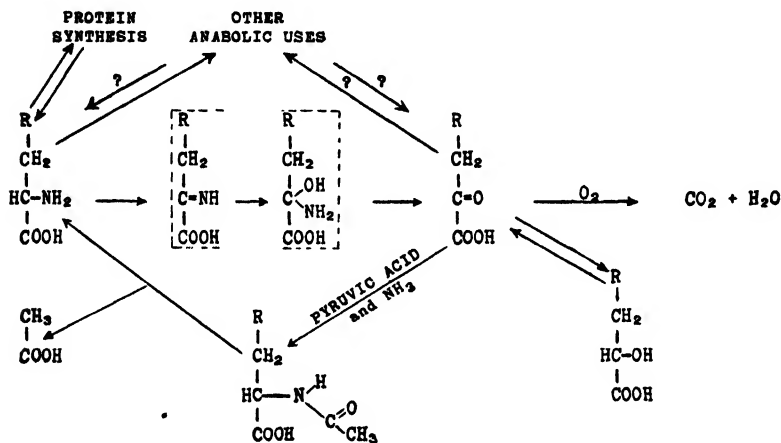
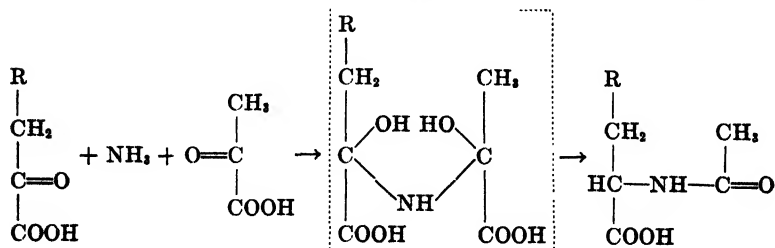
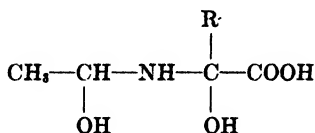


FIG. 2

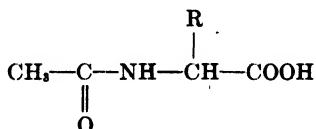
As indicated in this outline, the oxidative deamination is not directly reversible but the amino acid is resynthesized by a coupled oxidation-reduction involving pyruvic acid and ammonia. This latter reaction may take place perhaps *in vivo* through the formation of the same intermediate postulated by Erlenmeyer and Kunlin (11, 12) and by de Jong (13) for the *in vitro* reaction.



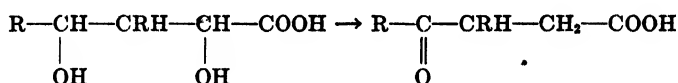
In the above hypothetical intermediate, after the splitting out of  $\text{CO}_2$ , we would have the arrangement



which according to Erlenmeyer and Kunlin (11) would be expected to rearrange with the loss of water to



This would be similar to the type of rearrangement studied by Erlenmeyer (25) and Erlenmeyer and Lux (26) of



The cycle of amino acid to keto acid, and the acetylamino acid to amino acid described above should, of course, be viewed as a dynamic one. One might visualize the amino acids being continually deaminized and resynthesized in the tissues, the equilibrium depending on the concentration and supply of the various reactants and particularly on the rate of removal of any participant in the cycle. If a demand for protein synthesis exists, the concentration of amino acid would naturally be decreased. On the other hand, if there is an influx of amino acid and not much demand for synthesis, the tendency toward oxidation would be augmented. The amount of amination of the keto acid would depend not only on the concentration of the latter, but on the pyruvic acid and ammonia available. Synthesis of amino acid should, therefore, be favored by an ample supply of carbohydrate. It may be here that carbohydrate and protein metabolism is tangential. It is interesting to recall the conclusion reached by Cathcart (27) many years ago from his studies on the protein-sparing action of carbohydrates, "that the carbohydrates are



absolutely essential for endo-cellular synthetic processes in connection with protein metabolism."

It is also of interest that one of the products of the series of reactions in this cycle would be acetic acid, and of particular significance is the fact that it would be formed at the expense of pyruvic acid. As was pointed out by Krebs (28), the formation of acetic acid from pyruvic acid by the acetyl mechanism would explain the formation of acetoacetic acid from ammonium pyruvate by minced liver observed by Annau (29) and by liver slices as found by Edson (30). Under the conditions of these latter experiments, any acetic acid formed in the acetyl cycle would be expected to yield acetoacetic acid (28).

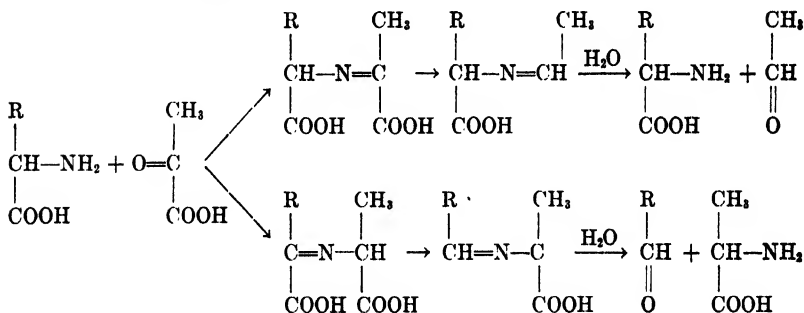
The recent work of Neber (31) and of Krebs (28) also lends much credence to the rôle that ammonia and pyruvic acid may play in amino acid synthesis. Neber was able to demonstrate that surviving rat liver can synthesize considerable amounts of amino nitrogen-containing compounds, presumably amino acids, when ammonium pyruvate is added. In work which confirmed Neber's results Krebs (28) brought forth the additional very interesting fact that, although increased amino acid nitrogen could be demonstrated after ammonium pyruvate, none could be demonstrated after other keto acids, such as ketoglutaric. This is particularly significant to the thesis of the present paper.

This type of reaction may also explain why the ammonium salt of phenylpyruvic acid yields acetoacetic acid in the presence of liver slices, whereas the sodium salt of phenylpyruvic acid does not (32). It may also be the explanation of why acetoacetic acid results from phenylalanine but not from phenylpyruvic acid (32). With phenylalanine the cycle is replenished at the amino acid stage and, if sufficient pyruvic acid is available, acetoacetic acid would be formed, whereas with phenylpyruvic acid sufficient ammonia would apparently not be available, just as in the corresponding results with pyruvic acid, alanine, and ammonium pyruvate. When the ammonium salt is given or the amino acid is given and ammonia formed by deamination, the acetoacetic acid would be formed.

With a naturally occurring amino acid the amount of acetylamino acid which would accumulate at any point would be negligible, since it probably would be quickly hydrolyzed to the free

amino acid. The cycle would be repeated and of course at the same time the oxidation of the keto acid would be taking place, thus gradually using up the supply of amino acid. With a homologue like phenylaminobutyric acid, the rate of oxidation of the keto acid is apparently so slow that the concentration of the keto acid would increase with the resultant increased synthesis of the acetyl amino acid, if sufficient pyruvic acid were available. Being compounds foreign to the body, both the keto acid and the acetyl amino acid tend to be excreted. The scheme well explains all of the observations of Knoop and of our own on phenylalanine, phenylaminobutyric acid, their acetyl derivatives, and the phenylketobutyric acid.

There is a high degree of similarity in this reaction of pyruvic acid, ammonia, and another keto acid to the reaction of pyruvic acid with amino acids which has been studied by Herbst (33). In this latter reaction an aldehyde and an amino acid were the end-products of the reaction. To account for their formation Herbst postulated the formation of a Schiff base, decomposing in one of two ways:



After the splitting out of  $\text{CO}_2$  a Schiff base is obtained which hydrolyzes with water to give acetaldehyde and an amino acid. The difference between this and the reaction of Erlenmeyer and de Jong is that no opportunity for the oxidation of the aldehyde portion of the molecule to an acid is possible; that is, there is no opportunity for a rearrangement in which one part of the molecule is oxidized at the expense of the other. It is quite clear that both Herbst's reaction and the reaction we are discussing might be formulated on the same type of intermediate. In Herbst's postu-

lation, water is split out to give the Schiff base, then a shift of the double bond may or may not take place,  $\text{CO}_2$  is liberated, and finally water is added again.

What we wish to emphasize is the similarity of these two reactions; we have pyruvic acid and an amino acid on the one hand yielding amino acid, acetaldehyde, and  $\text{CO}_2$ , while on the other hand we have a keto acid, ammonia, and pyruvic acid reacting to give an amino acid, acetic acid, and  $\text{CO}_2$ .

If, in the intermediate postulated by Erlenmeyer,  $\text{CO}_2$  is split off from the side of the molecule opposite that indicated in the equation above, we would get a fatty acid and alanine, in which instance the original keto acid is converted to the next lower fatty acid and pyruvic acid is converted to alanine. An interesting biological interrelationship between amino acids and fatty acids might, therefore, be indicated.

The reaction between a keto acid, ammonia, and pyruvic acid, resulting in the formation of an amino acid, is an interesting example of the so called coupled oxidation-reduction reactions. In this instance an intermediate is formed in which one part of the molecule is oxidized at the expense of the other part and after the resulting compound is hydrolyzed one compound is found to be oxidized and the other reduced.

#### SUMMARY

Evidence has been presented in favor of the reestablishment of Knoop's early acetyl theory for the *in vivo* synthesis of an amino acid from the corresponding keto acid, a theory which involves the reaction of the keto acid with pyruvic acid and ammonia, giving rise to the acetyl derivative of the amino acid as a normal intermediate step.

It has been shown that the body can oxidize the acetyl-*l*-phenylalanine more readily than the acetyl-*d*-phenylalanine, contrary to the conclusions of Knoop, and therefore the basis upon which Knoop abandoned his acetyl theory has been removed. Supporting data for the theory both from our own work and from the literature have been presented.

The direct demonstration of the biological conversion of one optical isomer into its enantiomorph in the animal body has been demonstrated. After the feeding of levorotatory phenylamino-

butyric acid it has been found that the acetyl derivative of the dextrorotatory form is excreted. The same acetyl derivative was found to be excreted after the feeding of the dextrorotatory phenylaminobutyric acid. The significance of these findings with respect to the acetyl theory of the synthesis of amino acids has been pointed out.

The synthesis and resolution of  $\gamma$ -phenyl- $\alpha$ -aminobutyric acid has been presented along with the demonstration that the dextrorotatory phenylaminobutyric acid corresponds in spatial configuration to the naturally occurring series of amino acids.

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## STUDIES ON THE CONSTITUTION OF INSULIN

### III. THE ACETYLATION OF INSULIN BY KETENE\*

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Ketene,  $\text{CH}_2=\text{C}=\text{O}$ , is admirably suited for the acetylation of proteins because it makes it possible to work in aqueous solution and at low temperatures; both of these factors aid in preventing secondary changes of the type of denaturation. The present experiments deal with the reaction of ketene with the primary amino groups and the phenolic hydroxyl groups of insulin. Sufficient amounts of the hormone were used to permit analytical studies of the resulting chemical changes as well as of the alterations in biological activity. This approach was suggested by the investigations of Northrop and Herriott (1, 2) on the acetylation of crystalline pepsin by ketene. The methods devised by the latter workers were found to be applicable, with slight modifications, to the present problem. It has been possible to demonstrate a striking similarity between the results of acetylation of crystalline pepsin and those obtained in the present study of insulin.

#### EXPERIMENTAL

*Insulin Preparation Used*—The insulin used in this investigation was the same as that previously employed in other experiments (3, 4); namely, highly purified, amorphous insulin, Lilly (20 to 22 units per mg.).

*Preparation of Ketene*—The ketene was generated by thermal

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decomposition of acetone vapor at heated platinum filaments (5), with the aid of the apparatus described by Herriott (6).

*Acetylation Procedure*—100 mg. of insulin were suspended in 10 cc. of *M* acetate buffer (pH 5.7) contained in the Pyrex glass chamber which has been used for the reduction experiments (3). For longer periods it was found necessary, in agreement with Herriott and Northrop (1), to replace the glass chamber by a dialysis thimble. Parchment membranes (Schleicher and Schüll) were found to be preferable to collodion sacks. The dialysis thimble was placed in a large volume (1 liter) of 3 *M* acetate buffer. In the course of the acetylation, the initial hydrogen ion concentration (pH 5.7) increases owing to the interaction of ketene with water to form acetic acid. The pH of the initial and of the final experimental mixtures was measured with the glass electrode.<sup>1</sup>

*Recovery of Acetyl Insulin*—At the end of the period of ketene treatment, the insulin suspensions were centrifuged. The acetylated product was repeatedly washed at the centrifuge with acetone and with ether, and dried *in vacuo* over sulfuric acid.

### Methods

The amino nitrogen was determined with the Van Slyke gasometric apparatus, with the micromodification of Koch (7). Tyrosine and O-acetyl content were determined by the methods described by Herriott (2). The tyrosine content is found by comparing the acetylated preparations with the original insulin, without previous hydrolysis. The O-acetyl content is determined by the comparison of the results of the so called "pH 8 and pH 11 methods" of Herriott. These procedures are based on the fact that acetyl groups attached to phenolic hydroxyls, in contrast to acetyl radicals attached to primary amino groups, are removed by a short hydrolysis at pH 11. The modification employed in the present work consisted in adding urea to the color mixtures to make a total urea concentration of 6 *M*; this was necessary in order to keep the protein in solution. Furthermore, the color was permitted to develop for 5 to 6 hours at room temperature before measurement, rather than for 15 minutes at 37° as recommended by Herriott. The color intensity was then measured with a

<sup>1</sup> Kindly determined by Mr. D. DuBois.

photoelectric colorimeter. The total nitrogen determinations were carried out by the Kjeldahl method, with the semi-micro-modification described by Hitchcock and Belden (8).<sup>2</sup>

*Bioassay Method*—The mouse convulsion method was used. Preliminary assays on small groups of mice by the method of Trevan and Boock (9) were carried out in this laboratory. The more important products were then assayed by the same method under the supervision of Mr. G. B. Walden in the Lilly Research Laboratories in Indianapolis.

### Results

*Time Course of Acetylation*—The amino nitrogen content of the insulin preparation used in this work was found to be 0.87 per cent. When corrected for moisture (5.76 per cent), this value becomes 0.92 per cent. If a molecular weight of 35,000 is assumed for insulin (10), this amino nitrogen value corresponds to twenty-one free amino groups per molecule of the protein. The tyrosine content of insulin is 12 per cent (11), from which it may be calculated that there are twenty-three phenolic hydroxyl groups, as tyrosine, per hormone molecule. In agreement with the results of previous workers on amino acids (12) and on crystalline pepsin (1, 2), it was found that the amino groups in insulin react with ketene much more rapidly than do the hydroxyl groups. In the course of the first few minutes of exposure to the reagent under the conditions employed, all of the  $\text{NH}_2$  groups are blocked, whereas no appreciable change in the number of the free OH groups can be detected. Upon continued treatment with ketene the OH groups are slowly acetylated. The acetylation of the latter groups in insulin is practically complete after 20 hours under the conditions described.

Some typical results obtained by varying the time, and in some cases also the temperature, of the insulin-ketene interaction are shown in Table I. While most of the values given in Table I are self-explanatory, the manner of representing the tyrosine content of the acetylated preparations requires an explanation. Most of the preparations, after recovery, contained more or less foreign, non-protein material (probably acetate) in the form of a loose

<sup>2</sup> Acknowledgment is made to Miss Alice C. Taylor for these nitrogen determinations.



addition complex.<sup>3</sup> Furthermore, the color given by the samples with Folin's reagent, when compared with the color developed by pure tyrosine standard solutions, yields tyrosine values lower than those obtainable after complete hydrolysis of the protein. For these reasons the tyrosine content as indicated by the pH 11 method, *i.e.* after the O-acetyl groups are split off, has significance only when it is referred to the result obtained with the pH 8 method, which leaves the O-acetyl groups intact. The ratio of the two sets of data affords a means of determining the extent of acetylation of phenolic hydroxyl groups in the prepara-

TABLE I  
*Representative Data for Acetylated Insulin Preparations*

Sample No.	Acetylation			Amino nitrogen	Amino groups substituted (total, 21)	Tyrosine ratio, pH 8 to pH 11 value	Hydroxyl groups substituted (total, 23)
	Time	Temperature	Final pH				
	<i>min.</i>	<i>°C.</i>		<i>per cent</i>			
11	5	24	5.68	0	21	0.96	0-1
15	10	1		0.38	12-13	0.93	1-2
3	10	25	4.73	0.09	18-19		
9	10	22	5.29	0	21	0.89	2-3
17	15	1	5.49	0	21	0.88	2-3
2	20	25.5	4.58	0	21		
7	30	25	5.14	0.26	15		
8	45	23	4.65	0	21	0.7	7
19	120	1	4.47	0	21	0.68	7-8
21	360	20-22	5.7	0	21	0.43	13
22	1080	23-27	4.66		21	0.36	14-15
23	1800	23-27	5.27		21	0.13	20

tions. The figures given under the heading tyrosine represent the ratio of the chromogenic values obtained by the two methods and are, therefore, equal to that fraction of the tyrosine of the insulin which has not been acetylated.

The data in Table I demonstrate an influence of temperature on the reaction rate. While a period of 5 minutes at room temperature (23-25°) was sufficient to block all of the free amino groups, a

<sup>3</sup> A similar experience has been reported by Charles and Scott (13). The foreign material is readily removed by dialysis at low temperature.

treatment for 10 minutes at 1° left about one-third of the amino groups untouched.

It may be seen from Table I that preparations were obtained in which all of the free amino groups but no appreciable number of hydroxyl groups (zero to three) had been acetylated. This fact demonstrates clearly the suitability of ketene for the problem at hand. The extent of acetylation of the phenolic hydroxyl groups becomes appreciable only after ketene treatment for at least 45 minutes. The slow rate of this process permits any desired gradation of the extent of acetylation. After 6 hours a little more than half of the hydroxyl groups have been blocked. Further acetylation of these groups then becomes still slower. After 30 hours, most of the phenolic hydroxyl groups are substituted.

*Properties of Acetyl Insulin Preparations*—The acetylated insulin preparations obtained in the course of this work, with the exception of the long term acetylated Sample 23, were all water-soluble. The lack of solubility of Sample 23, and, incidentally, of the acetyl preparations described previously, appears to be due to denaturation of the protein rather than to the acetylation process itself.

Crystallization of some of the amino-acetylated insulin preparations (Samples 10 and 17) by the zinc method (14) or by the pyridine-acetic acid method (11) yielded microcrystalline preparations with a crystalline form similar to that of non-acetylated, crystalline insulin.

*Physiological Activity of Acetyl Insulin Preparations*—The figures obtained by Mr. G. B. Walden in the Lilly Research Laboratories are given in Table II. These data demonstrate that the blocking of the free amino groups of insulin by acetyl radicals does not appreciably alter the physiological activity of the protein. In contrast to this result, with increasing substitution of the phenolic hydroxyl groups of the tyrosine in insulin, the activity decreases. General experience has shown that N-acetyl groups, in contrast to O-acetyl groups, are very stable and that they are not split off by moderate concentrations of acids or alkali. There is no reason to assume that the high activity of the amino-acetylated products is due to rapid hydrolysis in the organism. *The findings suggest that the free amino groups of insulin play no significant*

*rôle in its pharmacodynamic action. On the other hand, the phenolic hydroxyls of tyrosine in the hormone appear to be of importance for its biological activity.* Under the conditions of the biological test used, *i.e.* the acute convulsion method, the acetyl radicals attached to the hydroxyl groups are obviously not removed from the hormone molecule. Inasmuch as the tissues contain an enzyme (choline esterase) capable of splitting O-acetyl groups from compounds, the possibility exists that the acetyl radicals attached to the phenolic hydroxyls may eventually be split off. A delayed physiological action might, then, be expected from such preparations under other conditions of assay. Sample 22 was assayed on five rabbits by Mr. Walden. Although the number of animals

TABLE II

*Physiological Activity of Acetyl Insulin Preparations*

Approximately 60 mice were used in each assay.

Preparation No.	Amino groups acetylated	Hydroxyl groups acetylated	Physiological activity	Physiological activity, corrected for purity
	<i>per cent</i>	<i>per cent</i>	<i>units per mg.</i>	<i>units per mg.</i>
Insulin, Lilly.....	0	0	20-22	20-22
17. Acetyl insulin.....	100	0	13-15	17-19.5
22. " ".....	100	63	6	6
23. " ".....	100	87	0.5	0.6

used is very small, there was no indication of any delayed action and the sample appeared to have the same activity (somewhat less than 6 units per mg.) as was found when the mouse convulsion method of assay was employed.

## DISCUSSION

The acetylation of insulin has been studied by a number of investigators (13, 15-19), using acetic anhydride in various solvents. In general, two properties were characteristic of practically all of the acetylated insulin preparations which were obtained by the above procedures. First, the products were insoluble in water. Secondly, all preparations still contained some free amino nitrogen. No analytical determinations of phenolic hydroxyl

groups were made. The physiological activity was greatly diminished and could partly be regenerated by treatment with dilute alkali. It is obvious that the products obtained by means of acetic anhydride were partly amino- and partly hydroxyl-acetylated derivatives of insulin. The lack of solubility in water suggests, moreover, that some denaturation had occurred.

The statement that the free amino groups of insulin are indispensable for its action has been based largely on the inactivation of the hormone by phenyl isocyanate. It has been reported (20, 21) that phenyl isocyanate reacts only with primary amino groups in insulin. To strengthen this view, Gaunt and Wormall (22) recently conducted model experiments with a number of amino acids. In all cases the amino groups were substituted. No reaction could be detected with the hydroxyl groups of tyrosine. Gaunt and Wormall feel that "no evidence has been obtained which necessitates modification of the view previously expressed that phenyl isocyanate reacts only with the  $\epsilon$ -amino groups of lysine and any free  $\alpha$ -amino groups present in proteins." One is faced, therefore, with the statements, on the one hand, that the free amino groups in insulin are essential for its hypoglycemic action (20-22) and, on the other hand, with the evidence offered in the present paper which indicates that the contrary is true. Further work appears to be necessary to decide between the two opposing views. It might be pointed out, however, that the evidence offered in the past is largely of an indirect nature. No analyses have been published for the phenyl carbamido-insulin derivatives with respect to their content of phenolic hydroxyl groups. Experiments of the type performed by Gaunt and Wormall cannot be accepted without reservation in so far as their bearing on the insulin problem is concerned.

It is of interest to compare the results of the present work with those obtained by Herriott and Northrop on the acetylation of pepsin. In both investigations, pure proteins of highly specific, though different, biological activity were allowed to react with the same reagent, ketene, under carefully controlled conditions. In both instances the resulting products were analyzed and their biological activity determined. The methods employed in the chemical analyses were the same. These circumstances permit a comparative analysis of the results, free from distortion by second-

ary factors. The primary amino groups are essential neither for the proteolytic action of pepsin nor for the hypoglycemic action of insulin. On the other hand, the hydroxyl group of the tyrosine component of insulin or of pepsin appears to play an important part in the activity of these two biocatalysts. The decrease in activity resulting in both cases from acetylation of the phenolic hydroxyl groups depends on the extent of substitution of these groups.

#### SUMMARY

The reaction between insulin and ketene has been studied. Depending on the experimental conditions chosen, two types of acetyl derivatives of insulin have been obtained. If the time of interaction is very short (of the order of minutes), only free amino groups are acetylated. This process is complete after treatment of insulin with ketene at pH 5.7 for 5 minutes at room temperature. When the reaction is allowed to continue beyond this time, the hydroxyl groups of the tyrosine of insulin are slowly acetylated. This second phase of the process is practically complete only after 30 hours. The content of the acetylated preparations in free amino nitrogen and in free phenolic hydroxyl groups has been determined.

With the exception of the preparation obtained by ketene treatment lasting 30 hours, the acetylated insulin preparations were found to be readily soluble in water. Preparations in which all of the free amino groups but no appreciable number of hydroxyls had been blocked could be obtained in microcrystalline form by the methods ordinarily employed for the crystallization of insulin.

The physiological activity of the acetyl insulin preparations was determined by the mouse convulsion method. It was found that the acetylation of the free amino groups of insulin does not appreciably affect its pharmacodynamic action. When the tyrosine hydroxyl groups of the hormone are acetylated, the activity decreases with an increase of the number of blocked groups.

It is concluded that the free amino groups of insulin are dispensable for its physiological activity, whereas the tyrosine hydroxyl groups play an important rôle in the action mechanism of the hormone. These results are analogous to those obtained by Northrop and Herriott in a study of the acetylation of pepsin.

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## THE RELATION OF FAT TO THE UTILIZATION OF LACTOSE IN MILK\*

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In 1932 it was demonstrated in our laboratory (1) that rats placed on whole cow's milk mineralized with iron, copper, and manganese not only made excellent growth but used the milk solids very efficiently. Only 2.25 gm. of milk solids were required to produce 1 gm. of gain in weight. Since milk solids contain about 40 per cent of lactose and since lactose is not metabolized as rapidly as glucose, we were interested in the ability of rats to handle such large amounts of this sugar. It is impossible to discuss here, even in a general way, the literature on the metabolism of lactose and galactose. The more recent work has been reviewed by Pierce (2) and by Deuel (3).

When we started our work, we were aware of only two studies in which high levels of lactose or galactose had been used in the diet of rats. Mitchell and Dodge (4) had produced cataract in rats by feeding a synthetic diet containing 70 per cent of lactose. Guha (5) had reported that rats placed on a fat-free diet containing galactose as the sole carbohydrate rapidly decreased in weight and died.

We had never seen cataract in rats reared on mineralized milk, but of course a whole milk diet is quite different from a synthetic diet high in lactose. A more complete study of the metabolism of rats on milk diets was therefore undertaken.

### EXPERIMENTAL

Young albino rats about 28 days old were used for these studies. The rats were placed in separate metabolism cages which were so

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designed that the urine and feces were collected separately and quantitatively. Fresh whole milk was fed to each rat every morning and evening from a dish which was attached to the cage in such a manner that the rat did not spill any of the milk into the metabolism cage. The milk was supplemented with ferric pyrophosphate, copper sulfate, and manganese sulfate in such amounts that each 100 cc. of milk contained 1.5 mg. of elemental iron, 0.15 mg. of elemental copper, and 0.15 mg. of elemental manganese. These animals were kept on the whole milk diet for several weeks and during this time the urine and feces were collected each day and tested for reducing sugars with Benedict's solution. The urine was usually collected over chloroform to prevent as much fermentation as possible and boiled to remove the chloroform before the test was applied. The feces were soaked in water and a test for reducing sugars was made on the water extract. These animals very seldom showed more than a trace of reducing substances in their urine and no trace of reducing substances in their feces. Thus rats receiving their entire energy supply from whole milk utilized the lactose in the milk completely.

Since skim milk is commonly used as a food and since the lactose content of skim milk is somewhat higher than that of whole milk, it was thought advisable to run similar metabolism tests on rats receiving skim milk. The skim milk was supplemented with minerals in the same manner as the whole milk and in addition sufficient carotene was added so that each rat received 5 micrograms per day. These animals were also irradiated 10 minutes each day. After the animals had been on the skim milk diet for a few days, a positive test for reducing substances in the urine was obtained. When the animals were changed back to the whole milk diet or fed butter along with the skim milk, the reducing substances disappeared from the urine. No reducing substances were found in the feces of the animals on the skim milk except in cases of severe diarrhea.

A further study of this phenomenon was then undertaken to determine: (1) the kind and amount of sugar in the urine, (2) the constituent in butter fat active in preventing the loss of sugar, (3) the blood sugar picture of the animals. The rats taken for this work were started on a whole milk diet soon after weaning and kept on it until they were about 10 weeks of age. At this

time they were transferred to the skim milk diet and restricted to about 60 or 70 cc. per day, of which half was fed in the morning and half in the evening. The animals were restricted to this amount so that when the fat supplement was given they would continue to consume the usual amount of milk and consequently the same amount of milk sugar. The fat supplements and other compounds were fed in a small dish separately from the milk whenever possible. 24 hour metabolism tests were made two or three times weekly and a record kept of the milk consumed and the urine excreted. Sugar determinations were made according to a modified Shaffer-Hartmann method (6) and calculated as galactose. We standardized this method against pure galactose for our use. It was found then that  $\text{galactose} = \text{glucose} \times 1.22$ .

From the data obtained we calculated the amount of sugar consumed and excreted by each animal. It was found that the urine from the animals on the skim milk diet usually contained about 0.5 to 1 per cent galactose. This loss amounted to about 9 to 18 per cent of the total ingested milk sugar or 18 to 36 per cent of the ingested galactose. We also placed rats on the skim milk diet *ad libitum* and found that these animals lost a similar amount of sugar in their urine. Results which are typical of all animals studied are given in Table I.

*Identification of Sugar*—The sugar occurring in the urine of the animals on the skim milk diet was identified as galactose. Upon boiling the urine with nitric acid an insoluble compound was obtained which was identified as mucic acid. The recrystallized acid melted at  $208^{\circ}$  uncorrected and had a neutralization equivalent of 112. Mucic acid prepared under similar conditions from pure galactose had a melting point of  $208^{\circ}$  and a neutralization equivalent of 111. Characteristic galactosazones were obtained from the urine sugar after the urine was clarified with mercuric acetate. The melting point of the galactosazone was  $182\text{--}184^{\circ}$ , while the melting point of the osazone obtained from pure galactose and prepared under similar conditions was  $185^{\circ}$ . No part of the reducing material disappeared from the sterilized urine inoculated with a pure inoculum of *Saccharomyces cerevisiae*. This sample of yeast fermented glucose readily but did not ferment galactose. All of the reducing material disappeared from the sterilized urine when it was inoculated with *Torula galactosa*, a

TABLE I  
Data Obtained on Whole Milk, Skim Milk, and Skim Milk Plus Fat Diets

Rat 28					Rat 32					Rat 24				
Date	Milk	Urine	Galac- tose in urine	In- gested galac- tose lost in urine	Date	Milk	Urine	Galac- tose in urine	In- gested galac- tose lost in urine	Date	Milk	Urine	Galac- tose in urine	In- gested galac- tose lost in urine
Whole milk					Whole milk					Whole milk				
1936	cc.	cc.	per cent	per cent	1936	cc.	cc.	per cent	per cent	1937	cc.	cc.	per cent	per cent
Feb. 19	49	26	None	0.0	Feb. 19	40	20	None	0.0	Mar. 18	45	24	0.14	1.6
" 25	54	33	"	0.0	" 25	40	19	"	0.0	" 30	58	35	None	0.0
Mar. 3	41	22	"	0.0	Mar. 3	30	15	"	0.0					0.0
" 4	50	23	"	0.0	" 4	40	20	"	0.0					
" 11	70	35	"	0.0	" 11	37	22	"	0.0					
" 19	67	34	"	0.0	" 19	38	20	"	0.0					
					" 26	30	16	"	0.0					
					Apr. 2	61	31	"	0.0					
					" 9	40	24	"	0.0					
Skim milk					Skim milk					Skim milk				
Mar. 22	70	50	0.29	4.1	Apr. 13	85	64	0.50	7.5	Apr. 6	60	47	0.44	6.9
" 26	70	54	0.70	10.8	" 16	110	90	0.53	8.7	" 11	60	51	0.64	10.8
" 30	70	54	0.74	11.4	" 23	125	97	0.57	8.9	" 14	60	51	0.77	13.1
Apr. 2	70	57	0.71	11.6	" 27	125	97	0.60	9.3	" 19	60	46	0.74	11.4
" 9	80	57	0.82	11.7	" 27	125	97	0.60	9.3	" 23	60	50	0.87	14.5
" 13	80	63	0.85	13.4	May 11	100	80	0.77	12.3	" 28	60	50	0.76	12.7
" 16	80	67	0.73	12.5	" 20	100	84	0.96	16.1	" 30	60	54	0.72	13.0
" 23	80	70	0.91	15.9	June 2	103	90	0.80	14.0	May 3	60	55	0.59	10.8
" 27	80	63	1.10	17.3	" 9	105	90	0.85	14.6	" 5	60	52	0.67	11.6
" 28	80	69	0.82	14.1										
										May 7. Plus 4 per cent linseed oil				
Plus 4 per cent corn oil					Apr. 13	85	64	0.50	7.5	May 12	60	42	0.20	2.8
May 5	80	57	0.11	1.5	" 16	110	90	0.53	8.7	" 14	60	47	0.14	2.2
" 8	70	50	None	0.0	" 23	125	97	0.57	8.9	" 17	60	42	0.16	2.2
" 13	80	57	"	0.0	" 27	125	97	0.60	9.3	" 19	60	46	None	0.0
" 18	70	56	"	0.0	May 11	100	80	0.77	12.3	" 27	60	45	"	0.0
					" 20	100	84	0.96	16.1	" 28	60	49	"	0.0
					June 2	103	90	0.80	14.0	" 31	60	47	"	0.0
					" 9	105	90	0.85	14.6					

galactose-fermenting yeast. This indicates that all of the sugar in the urine was galactose and that the problem concerned is one of galactose metabolism.

*Action of Fat*—Our investigations on the relation of the fat to the utilization of the milk sugar showed that the natural fat content of the milk, 3 to 4 per cent, is necessary to enable the animal to make complete utilization of the galactose. It was also found that other fats, such as lard and corn oil, could be substituted for the milk fat with equally good results. The fatty acid fraction of the fat molecule appears to be the active agent in the metabolism of galactose, since feeding glycerol along with the skim milk diet had no effect, while oleic acid was effective. It was somewhat difficult in most cases to feed the fatty acids in the free condition; consequently the triglycerides of oleic, palmitic, and caproic acids were made and fed with the skim milk diet. Tri-palmitin and triolein were found to be effective but tricaproin showed only a very slight effect if any at all. The animals receiving tricaproin with the skim milk diet gained weight upon the addition of the tricaproin to the diet, which indicated that the animals utilized the acid for some purpose but not for galactose metabolism. However, the animals which did not respond to tricaproin did respond when corn oil was added to the skim milk diet. We later tried feeding the sodium salts of butyric,  $\beta$ -hydroxybutyric, and lactic acids and found these to be ineffective. The sodium salts of lactic acid and  $\beta$ -hydroxybutyric acid were fed at levels of 2 per cent and the sodium salt of butyric acid at a level of 3 per cent. These amounts were as much as we could get the animals to take with the skim milk. However, when fats were fed at these levels, some response was always obtained. Other fats which were fed and found to be effective were coconut oil, hydrogenated coconut oil, and linseed oil. No difference could be detected between the saturated and unsaturated coconut oil. Glucose was found to have some influence on the utilization of the galactose with certain animals. This influence, however, may be limited to the animal's ability to synthesize fat from glucose. However, several workers (cf. Deuel (3)) have shown that absorption of galactose is influenced by the presence of glucose in the diet. Choline was also fed and found to be ineffective. The results are summarized in Table II. These data indicate that the lower

triglycerides are not effective in enabling the animal to utilize all of the galactose, while all of the fats and higher triglycerides fed were found to be effective.

*Blood Sugar Picture*—It was found that the blood sugar of the animals on the skim milk diet rose much higher after feeding than in the case of animals receiving whole milk or fat with the skim milk diet. The blood sugar of the animals on the skim milk diet rose from about 100 mg. to about 200 mg. per 100 cc. of blood, while the blood sugar of the animals receiving fat with their milk seldom rose higher than 140 mg., and no sugar was lost in the urine.

TABLE II  
*Effect of Various Fats, Fatty Acids, Etc., on Metabolism of Galactose*

Compound fed	Level fed	Result
Butter.....	4% milk diet	Effective
Lard.....	4% " "	"
Corn oil.....	4% " "	"
Linseed oil.....	4% " "	"
Coconut ".....	4% " "	"
" " hydrogenated....	4% " "	"
Oleic acid.....	4% " "	"
Triolein (synthetic).....	4% " "	"
Tripalmitin (synthetic).....	4% " "	"
Glucose.....	4% " "	Some with some animals
Tricaproin (synthetic).....	4% " "	Very slight, if any
Sodium lactate.....	2% " "	Not effective
" butyrate.....	3% " "	" "
$\beta$ -Hydroxybutyric acid.....	2% " "	" "
Choline.....	10 mg.	" "

The urine of the animals on the skim milk diet showed an increase in sugar content as the blood sugar rose, and fell again when the blood sugar returned to normal. This phenomenon was observed regardless of the rate at which an animal was allowed to drink its allotment of skim milk. High blood sugar was observed when a 30 cc. allotment of skim milk was fed, which was usually consumed in 2 to 3 hours, or when 30 cc. were fed at the rate of 2 cc. each half hour. In both cases the blood sugar rose soon after feeding was started and remained high until after all of the milk was consumed. Fig. 1 shows the blood sugar curves of two ani-

mals; both received skim milk at the rate of 2 cc. each half hour but one received coconut oil along with the skim milk diet. The percentage of sugar in the urine collected over 2 hour periods is also shown in Fig. 1. The blood sugar determinations were carried out according to the method of Benedict (7) and modified slightly so that smaller amounts of blood could be used. This was

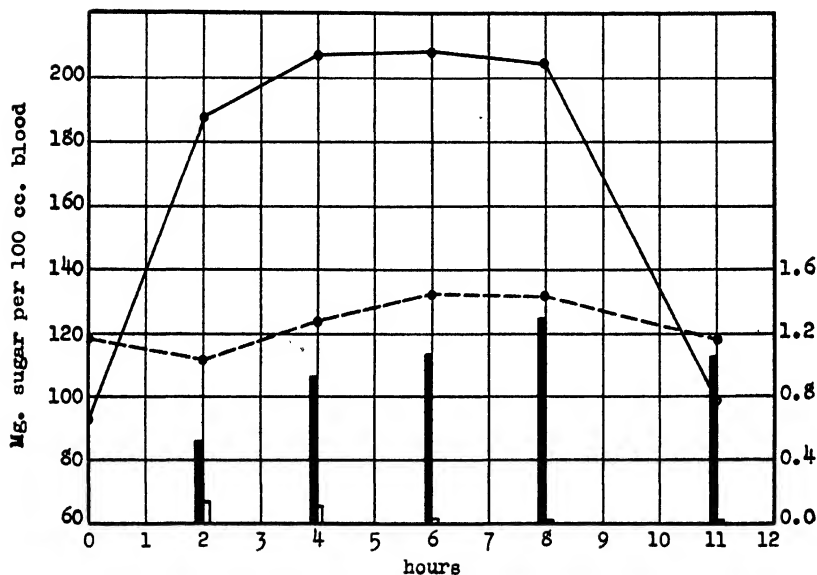


FIG. 1. Blood sugar curves for rats on skim milk and skim milk plus fat diets. Each animal received 30 cc. of skim milk fed at the rate of 2 cc. each half hour. The continuous line represents the blood sugar curve of Rat 242 on the skim milk diet; the dash line, the blood sugar curve of Rat 243 on the skim milk diet plus coconut oil; the solid perpendiculars indicate the percentage of sugar in the urine of Rat 242; the clear perpendiculars, the percentage of sugar in the urine of Rat 243.

necessary in order to make frequent blood sugar determinations during the day. While the sugar in the blood was not characterized, the excessive high blood sugar was presumably due to galactose since galactose was being excreted into the urine.

A similar experiment was carried out with a pig and a calf. Both of these animals made complete utilization of the milk sugar on a whole milk diet, but after about 2 weeks of skim milk feeding

considerable amounts of galactose began to appear in the urine of these animals. The pig lost about 6 per cent of the total amount of lactose ingested or approximately 12 per cent of the ingested galactose. The calf lost about 8 per cent of the total lactose ingested or approximately 16 per cent of the total galactose ingested. When 4 per cent of corn oil was added to the skim milk diet of the pig, an immediate decrease of sugar in the urine was observed and after 2 weeks no sugar could be detected. Both of these animals responded much more slowly to a change in the diet than did the rats.

#### DISCUSSION

These experiments have shown that some relation exists between the presence of fat in the milk and the efficient utilization of the galactose part of the lactose molecule. The animal body apparently can utilize the glucose fraction of the lactose molecule without difficulty but is unable to utilize the galactose fraction efficiently in the absence of fats. The fact that sugar was found in the urine of the animals on the skim milk diet is readily explained when the high blood sugar is taken into account, but just why the presence of fat in the milk prevented an abnormal rise in blood sugar cannot be explained at present. We have fed dry synthetic rations which were fat-free containing glucose, lactose, or galactose and found that on the glucose ration the animals utilized the sugar very well, while on the lactose or galactose rations considerable amounts of galactose appeared in the urine. This further supports the evidence that galactose is the sugar concerned in this phenomenon. Addition of fat to the synthetic lactose ration to the extent of 35 per cent stopped the loss of most of the sugar but complete utilization was not brought about by addition of fat to the synthetic galactose ration. In the latter case the amount of free galactose in the intestine may have been too high for the amount of fat present and the galactose passed into the blood stream faster than it could be taken care of by the animal. On the other hand, the galactose liberated from lactose in the intestine may be such that the fat can take care of all of the galactose. Whether the fat merely acts to slow up absorption or takes a part in the conversion of galactose into a metabolizable form cannot be stated at present. It appeared to us at

first that the action of fat was in slowing up the absorption of sugar from the intestine, but when the skim milk was fed at the rate of 2 cc. each half hour the animals still showed an abnormal rise in blood sugar and still lost as much or more sugar in the urine as when 30 cc. were fed at one time, which were usually consumed in 2 to 3 hours. Another point which does not support the hypothesis of delayed absorption is the fact that after the fat was removed from the milk diet, the animals continued to utilize all the sugar for a few days. However, it is possible that the animal may have secreted fat into the intestine to aid in galactose metabolism. We believe from this work that the fat plays some part in the metabolism of lactose and galactose other than possibly decreasing the rate of absorption.

It has been pointed out by a number of workers that galactose has a different course in its metabolism than glucose. Deuel, Gulick, and Butts (8) and Clark and Murlin (9) found that galactose is superior to other hexoses in ketolytic activity. Cori (10) has found that absorption of galactose from the intestine takes place somewhat faster than glucose and that glycogen is formed from galactose much more slowly than from glucose. Bell (11) and Harding, Grant, and Glaister (12) have shown that glycogen formed from galactose has a different structure than glycogen formed from glucose, but glucose results from the hydrolysis of both forms of glycogen. Deuel, Hallman, Butts, and Murray (13) have pointed out that fatty acids containing 8 or more carbon atoms are much better ketone body producers than butyric and caproic acids. We found butyric and caproic acids to be ineffective in preventing a loss of galactose in the urine. The significance of the above work in its relation to our own cannot be evaluated until further work on the action of specific fatty acids on galactose metabolism has been carried out.

#### SUMMARY

1. Rats placed on a mineralized *whole* milk diet made very efficient utilization of all of the milk sugar. This has been found to be true for a pig and a calf.

2. When the animals were placed on a mineralized *skim* milk diet, sugar was readily detected in the urine after a few days of feeding. The sugar was identified as galactose and accounted for



all of the reducing material in the urine. In the case of the rat as high as 35 per cent of the ingested galactose was recovered in the urine.

3. Fats such as butter fat, lard, corn oil, coconut oil, linseed oil, and palmitic and oleic acids when added to the mineralized skim milk at levels of 3 to 4 per cent, prevented this loss in the urine. Glycerol or butyric,  $\beta$ -hydroxybutyric, caproic, and lactic acids did not prevent the loss.

4. On mineralized skim milk the sugar of the blood rose to about 200 mg. per cent, while on whole milk it seldom rose higher than 140 mg. per cent after feeding.

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## A NEW TYPE OF ABSORPTION CELL FOR THE PHOTOELECTRIC MICROCOLORIMETER\*

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In adapting the blood volume technique of Gibson and Evans (1) to the photoelectric colorimeter of Evelyn and Cipriani (2), the necessity for filling the open absorption cells with an exactly pipetted volume of serum proved to be a serious source of inconvenience. In addition it was found that significant errors were introduced by aberrations in the shape of the meniscus obtained when the cells were filled with undiluted serum. Although the completely closed cell would have eliminated the meniscus error, the unavoidable inconvenience of filling and cleaning such cells encouraged us to design a plunger type cell which would combine the advantages of both open and closed types. The performance of this new cell has proved so satisfactory that it has been adopted for most of the new microcolorimetric procedures and has resulted in a considerable improvement in the convenience and accuracy of the apparatus as a whole.

A cross-sectional drawing of the new cell is shown in Fig. 1. The glass portion is similar to the 2.0 cc. open cell described in the original paper (2), being made from precision bore tubing (11.28 mm. inside diameter) with a fused or cemented plane glass bottom. Into the top of this cell there is accurately fitted a removable metal cap which carries a clear glass plunger with plane-parallel ends and a coating of opaque material on the sides. The lower 2 or 3 mm. of the plunger dip into the sample solution, and the thickness of the layer through which the light beam passes is

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accurately defined by the distance between the lower surface of the plunger and the base of the cell. The plunger is held in place in the metal cap by means of a set screw, and the thickness of the absorbing layer can be varied at will by use of a plunger of suitable length. The three most commonly used plungers are adjustable for depths of 10.0, 5.0, and 1.0 mm. respectively, the corresponding minimum sample volumes being 1.1, 0.6, and 0.15 cc. The inside height of the cell, the thickness of the metal cap, and the length of the plungers are specified accurately so that the correct heights are obtained automatically but as an added precaution a precision steel gage block of appropriate height may be placed in the bottom of the cell and the set screw in the cap

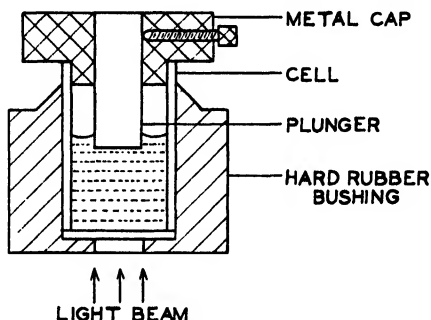


FIG. 1. Cross-sectional drawing of cell with metal cap and plunger in place. The cell is shown mounted in the removable hard rubber bushing which fits into the socket of the sliding carriage of the microcolorimeter.

tightened while the plunger is pressed firmly against the gage. This adjustment is only necessary at infrequent intervals, since the plunger and cap are removed as a unit and are seldom taken apart even for cleaning, as only the lower end of the plunger comes in contact with the sample solution.

The sample solution may either be made up directly in the cell, or may be transferred to it by means of a bulb pipette. A series of rings etched around the cell serves as a guide for insertion of approximately the correct amount of fluid, although an excess of solution does no harm unless the level rises high enough to come in contact with the metal cap. It is so easy to clean the bottom of the plunger after each reading that it is usually convenient to

use the same plunger and cap for all members of a series of similar solutions.

TABLE I

*Comparison of Results of Measurements on Same Solution in Different Absorption Cells*

All concentrations are given in arbitrary units to the nearest 0.5 per cent. The results obtained with cells of different thicknesses have been reduced to a standard thickness of 10.0 mm. by multiplying by the appropriate factor. In the case of measurements made on the macrocolorimeter with the test-tube type of absorption cell, the thickness of the absorbing layer has been taken to be 19.2 mm., which is the calculated value of the thickness of an equivalent plane-parallel cell. The agreement between the second and third columns proves that the error involved in treating the tubular cell as though it were a plane-parallel cell of 19.2 mm. internal thickness does not exceed  $\pm 0.5$  per cent. This offers further proof of the fact that photoelectric measurements made through a tubular cell are in all respects analogous to those made through conventional plane-parallel cells. Moreover, the greatly increased convenience of the tubular cell is attained without any sacrifice of accuracy or precision, as shown by the agreement of the triplicate readings made in each case.

Solution	Measurement made in test-tube cell of macrocolorimeter, $\times 1.92$	Measurement made in plunger type microcell at 10.0 mm. depth	Measurement made in micro-cell at 5.0 mm. depth, $\times 2$	Measurement made in micro-cell at 1.0 mm. depth, $\times 10$
Oxyhemoglobin in water	100.0	100.0	101.0	102.0
	100.0	100.5	100.5	100.0
	99.5	100.5	100.5	100.5
Naphthol green in water	100.0	99.5	99.0	98.5
	100.0	100.5	100.5	101.5
	100.0	100.0	99.5	99.5
"Evans blue" dye in serum	100.5	100.0	98.5	97.5
	100.5	100.5	99.5	100.5
	99.5	101.0	99.0	100.0
Azobilirubin in alcohol	100.0	99.5	100.5	100.5
	100.0	100.0	101.0	102.5
	100.5	100.5	101.0	99.0
Bilirubin in chloroform	99.5	101.0	98.5	102.5
	99.5	100.5	99.5	101.5
	100.0	99.5	100.5	99.0

A further advantage of the new cell, which only became apparent after it had been in use for some time, is that the adjustment of the liquid layer can be kept accurate down to such small thick-

nesses that the new cell may often be used to advantage in measurements on thin layers of very opaque solutions, instead of the somewhat cumbersome thin film cells which were employed formerly. Of course special cells still are required for measurements at thicknesses of the order of 0.5 mm. or less.

### Results

Table I illustrates the precision of the results obtained with the plunger cell and also the correlation of these results with those obtained on the original photoelectric macrocolorimeter (3) in which a test-tube type of absorption cell is employed. The figures show that measurements made with the plunger at any given depth may be converted to the corresponding values for a 10 mm. depth by assuming a strictly linear proportionality. Moreover, these figures can be compared directly with those obtained with the macrocolorimeter by assuming the effective depth of the test-tube cell to be 19.2 mm. This value has been checked carefully by comparative measurements on solutions of several different colors in various media such as water, serum, alcohol, and chloroform. The use of this conversion factor has proved very satisfactory in reducing to a common basis results obtained with the macro- and micromodifications of a number of techniques.

### SUMMARY

1. A new plunger type of absorption cell is described for use with the photoelectric microcolorimeter of Evelyn and Cipriani (2).
2. With the new cell measurements may be made on volumes of 1.1, 0.6, or 0.15 cc., the corresponding thicknesses of the absorbing layer being 10.0, 5.0, and 1.0 mm. respectively.
3. Data are presented to illustrate the accuracy of the results obtained with the new microcell as compared with measurements on the original photoelectric macrocolorimeter (3).

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## STUDIES ON THE REDUCTION OF DEHYDROASCORBIC ACID BY GUINEA PIG TISSUES\*

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It has been suggested frequently that one of the functions of ascorbic acid (vitamin C) in the animal organism may be that of a respiratory catalyst (1-6). The factors responsible for the postulated reversible oxidation and reduction of ascorbic acid by animal tissues have been poorly defined, however. Aside from the qualitative observation (1) that "hexuronic acid" slowly reduced cytochrome C, *animal tissue* constituents responsible for the oxidation of ascorbic acid, either directly or by catalysis, have not been identified. The reduction of dehydroascorbic acid by animal tissues, based on urinary excretion (4, 7) and tissue storage (8) of ascorbic acid after administration of dehydroascorbic acid, has been attributed to glutathione by Borsook *et al.* (4), although the possibility of enzymic reduction (9) has not been excluded.

The observation of Szent-Györgyi (1) that reversibly oxidized hexuronic (ascorbic) acid could be reduced by fixed —SH and glutathione has been followed by quantitative experiments by Hopkins and Morgan (3) and by Borsook *et al.* (4). The last two papers cited review the available evidence that tissues tend to protect ascorbic acid from oxidation. Recently Masayama and Tatematsu (10) reported that very small amounts of tissue could exert this protection and Yamamoto (11) found that *l*-adrenalin was similarly effective. Glutathione (12-14) in small quantities, boiled tissues (15), and other substances forming copper complexes (16) can inhibit the aerobic oxidation of ascorbic acid by

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copper catalysis. The fact that nearly all of the ascorbic acid in animal tissues occurs in the reduced form (5, 17) might be explained on the basis of either (a) protection against oxidation or (b) rapid reduction of the oxidized product.

#### EXPERIMENTAL

All experiments were carried out with tissues from normal guinea pigs. The tissues were removed after decapitation and bleeding, weighed, and rapidly frozen on blocks of solid  $\text{CO}_2$ . By grinding the tissues while frozen and during thawing after the addition of  $\text{m}/15$  phosphate buffer (pH 7.4), a homogeneous suspension was obtained. This could be readily pipetted and did not settle appreciably even upon prolonged standing. The suspension usually contained 1 gm. of fresh tissue in 5 ml. For ascorbic acid and glutathione analyses respectively, the tissue suspension was deproteinized with 6 per cent  $\text{CCl}_3\text{COOH}$  + 2 per cent  $\text{HPO}_3$ , and titrated with 2,6-dichlorophenol indophenol or 0.002  $\text{N}$  iodine in the presence of  $\text{KI}$ . The dye was standardized against crystalline ascorbic acid. Dehydroascorbic acid was prepared by oxidation with iodine. We realize that in this manner unphysiological quantities of iodide were introduced into the tissue suspensions. Borsook *et al.* (4) have shown, however, that the reduction of dehydroascorbic acid by glutathione is not affected by the nature of the oxidant. In our experiments ascorbic acid oxidized by iodine or by oxygen and copper gave practically identical results. All experiments involving the reduction of dehydroascorbic acid were carried out in Warburg respirometers under strictly anaerobic conditions in an atmosphere of 95 per cent  $\text{N}_2$  and 5 per cent  $\text{CO}_2$  (passed over freshly reduced hot copper turnings). The dehydroascorbic acid was added from the side arms to the main vessels after anaerobic conditions were established. At the end of the experiment the contents of the vessels were deproteinized and aliquots were titrated.

The data presented in Tables I to VI are, in all cases, based upon a considerable number of experiments, including the use of enough animals to avoid errors that might arise from using individual animals.

*Effect of Buffer (pH 7.4) on Dehydroascorbic Acid. Formation of a Reducing Substance*—In preliminary experiments carried

out aerobically we observed that dehydroascorbic acid added to phosphate buffer (pH 7.4) gave rise to a substance that reduced 2,6-dichlorophenol indophenol and iodine in acid solution. The formation of this reductant attained a maximum in 80 to 100 minutes, at which time it was equivalent to the reduction of 15 to 20 per cent of the dehydroascorbic acid added. After reaching this maximum, the reducing substance disappeared as illustrated in Table I. Herbert *et al.* (18) have noted the formation of this reductant, stating that "iodine however, is taken up to an extent consonant with the idea that the band at 245  $m\mu$  is due to ascorbic acid." They suggest that slightly alkaline solutions of dehydroascorbic acid undergo self-oxidation-reduction with partial regeneration of ascorbic acid. Moll and Wieters (19), however, state that the reducing substance is not ascorbic acid (veri-

TABLE I

*Formation of Indophenol-Reducing Substance during Aerobic Incubation of Dehydroascorbic Acid in Phosphate Buffer, pH 7.4, at 37.5°*

The results are expressed as per cent dehydroascorbic acid reduced to ascorbic acid. 20 ml. of buffer + 5 ml. of dehydroascorbic acid (10 mg.).

Min.....	20	40	60	80	100	120	140	160
Dehydroascorbic acid reduced. ....	7.8	15.5	20.6	20.9	20	13.2	8.8	1.6

fied by feeding tests). By working under anaerobic conditions the formation of this reducing material can be considerably decreased. In 30 minutes, the period over which most of our experiments extended, anaerobically, the indophenol titration was equivalent to a reduction of 2 to 3 per cent of the dehydroascorbic acid used. These observations emphasize the necessity of working under anaerobic conditions and of running blanks parallel with the experiments involving tissue.

*Instability of Dehydroascorbic Acid*—The instability of dehydroascorbic acid in physiological pH and temperature ranges has been stressed by Borsook *et al.* (4) and by Ball (20). Experiments confirming the irreversible nature of the change, and showing that it was not inhibited or accelerated by liver tissue, were carried out aerobically in the following manner:



Buffer or liver brei was added to aliquot portions of dehydroascorbic acid solution held at 37.5°, pH 7.4. At intervals the mixture was quickly deproteinized with 6 per cent  $\text{CCl}_3\text{COOH}$  + 2 per cent  $\text{HPO}_3$ , aliquots were added to 5 ml. of McIlvaine buffer, pH 3, and readjusted to this pH by the addition of dilute  $\text{NaOH}$ , with 2,4-dinitrophenol as an indicator. (We have been unable to obtain quantitative recoveries of iodine-oxidized ascorbic acid at pH values much less than 3.)  $\text{H}_2\text{S}$  was bubbled through the tubes for 90 minutes. They were then stoppered and left standing overnight. After the addition of sufficient  $\text{HPO}_3$  to bring the concentration to about 3 per cent,  $\text{H}_2\text{S}$  was removed from the solution by a stream of purified  $\text{CO}_2$ . The samples were then titrated with indophenol. Only about 60 per cent of the dehydroascorbic acid could be recovered after 3 minutes, and only about 40 per cent after 6 minutes.

*Reduction of Dehydroascorbic Acid Added to Liver. Effect of Iodoacetate and Arsenite*—Experiments extending over different periods of time indicated that the amount of dehydroascorbic acid reduced by liver brei was as great after 30 minutes as after 3 hours. In fact, within 3 minutes after the addition of dehydroascorbic acid to liver brei we have observed 70 per cent of the maximum reduction.

If glutathione is wholly responsible for the reduction of dehydroascorbic acid by animal tissues, it should be possible to inhibit the reaction by blocking the glutathione contained in the tissues. Dickens (21) has shown that the reaction between glutathione and iodoacetate proceeds rapidly at pH 7.4 and Mirsky and Anson (22) have used the same reaction to mask —SH groups in denatured proteins. If the reduction of dehydroascorbic acid were dependent on enzymes it would be abolished by heating. Table II illustrates the experimental basis for the following conclusions: (a) Anaerobically, liver brei markedly reduces dehydroascorbic acid added to it. (b) Incubation of liver brei with 0.01 M iodoacetate completely abolishes this reducing capacity. (Iodoacetate has no effect on ascorbic acid or on the indophenol titration.) (c) Heat-coagulated liver brei has a much greater reducing effect than unheated liver (on dehydroascorbic acid).

The amount of dehydroascorbic acid reduced by liver was found to vary from 10 to 18 per cent when 2 mg. of dehydroas-

corbic acid were added to 0.8 gm. of liver. When smaller amounts were added, the reduction could be increased to 30 or 40 per cent, although the absolute amount reduced was smaller. At the end of the reduction period the liver brei still contained much reduced glutathione, showing that the reduction of added dehydroascorbic acid by tissue glutathione was far from quantitative. This is in agreement with the observation of Borsook *et al.* (4) that complete reduction of dehydroascorbic acid by glutathione required a large excess of the latter.

Treatment of liver brei with 0.01 M sodium arsenite inhibited the reduction of dehydroascorbic acid completely. The same

TABLE II

*Reduction of Dehydroascorbic Acid Added to Liver Brei*

Anaerobic, 30 minutes, 37.5°. The liver brei in Flask 2 was heat-coagulated; that in Flask 3 treated with iodoacetate for 1 hour.

Contents	Flask 1	Flask 2	Flask 3	Flask 4
	ml.	ml.	ml.	ml.
Liver brei (0.8 gm. liver) . . . . .	4.0	4.0	4.00	0
H <sub>2</sub> O . . . . .	0.5	0.5	0.06	0.5
Iodoacetate, 0.1 M . . . . .	0	0	0.44	0
Buffer, M/15, pH 7.4 . . . . .	0	0	0	4.0
Dehydroascorbic acid, 2 mg. . . . .	0.5	0.5	0.50	0.5
	per cent	per cent	per cent	per cent
Dehydroascorbic acid reduced. . . . .	14.7*	38.2*	0*	1.7

\* These values have been corrected for ascorbic acid added with the liver and that produced in Flask 4.

results were obtained with glutathione prepared from yeast. The formation of thioarsinites by —SH compounds has been recorded frequently, and Cohen *et al.* (23) have reported the preparation of di(glutathionyl)acetanilide-*p*-thioarsinite. Voegtlin *et al.* (24, 25) have suggested that the pharmacological action of trivalent arsenic is essentially due to its reaction with glutathione and other —SH compounds in protoplasm.

*Reduction of Dehydroascorbic Acid by Heat-Coagulated Liver*—We have observed an increased reduction capacity after heat coagulation with liver, muscle, whole blood, and red blood cells, but not with blood plasma. Mirsky and Anson (26) have shown

that denaturation of protein may lead to the formation of —SH groups. If the reduction of dehydroascorbic acid by heat-coagulated tissues were due to acid-insoluble —SH groups, the reaction should be inhibited by iodoacetate or by preliminary oxidation of the —SH groups, as shown in the following experiments.

Liver brei (95 ml., 5 ml. per gm. of liver) was coagulated by heat, centrifuged, and the supernatant liquid discarded. The precipitate was washed for 30 minutes by a flow of distilled water delivered to the bottom of a tall cloth-covered flask containing the precipitate. The washed precipitate was suspended in  $M/15$  phosphate, pH 7.4, and made up to 95 ml. Reduction experiments were conducted with 4 ml. samples. The acid-soluble —SH of this suspension was only enough to give a maximum of 1 per cent reduction of dehydroascorbic acid. 50 ml. portions of washed coagulum were treated for 1 hour with 80 mg. of alloxan monohydrate, then washed as before with distilled water to remove the excess alloxan. The precipitate was again suspended in  $M/15$  phosphate of pH 7.4 and made up to 50 ml., 4 ml. samples being used for the reduction experiments. The results, given in Table III, show that heat-coagulated, washed liver tissue can reduce dehydroascorbic acid although it is practically free from acid-soluble reducing substances. This property, however, is abolished when the coagulum is treated with iodoacetate or alloxan before reduction. Purr (27) has used alloxan for the reversible oxidation of —SH groups in cathepsin. These observations clearly demonstrate that —SH compounds other than glutathione, namely fixed —SH, can reduce added dehydroascorbic acid.

*Reduction of Dehydroascorbic Acid by Other Tissues*—In addition to liver we have studied the reduction of dehydroascorbic acid by blood, red blood cells, blood plasma, muscle, and washed small intestine, and the effect of iodoacetate and heat coagulation thereon. From the results summarized in Table IV, it is evident that reduction was most extensive in the tissues having the highest glutathione content. In blood plasma, devoid of glutathione, no reduction was observed before or after heating. In all cases the reducing capacity of the tissues for dehydroascorbic acid could be completely eliminated by 0.01  $M$  iodoacetate, indicating

TABLE III

*Reduction of Dehydroascorbic Acid by Heat-Coagulated Liver*  
 Anaerobic, 30 minutes, 37.5°.

Contents	Flask 1	Flask 2	Flask 3	Flask 4
	ml.	ml.	ml.	ml.
Buffer, M/15 phosphate, pH 7.4....	4.5	0.5	0.5	0.06
Liver coagulum, washed	0	4.0	0	4.00
“ “ alloxan-treated..	0	0	4.0	0
Iodoacetate, 0.1 M.....	0	0	0	0.44
Dehydroascorbic acid, 2 mg.....	0.5	0.5	0.5	0.50
	per cent	per cent	per cent	per cent
Dehydroascorbic acid, reduced....	3.0	17.1	2.2	2.20

TABLE IV

*Reduction of 2 Mg. of Dehydroascorbic Acid by Various Tissues at pH 7.4, 37.5°, Anaerobic*

Time 30 minutes (120 minutes with whole blood and red blood cells). When iodoacetate was used, the tissues were treated with 0.01 M iodoacetate for 1 hour. The results were corrected for ascorbic acid present in the tissues and for that formed in the buffer.

Tissue	Amount of tissue used	Dehydroascorbic acid reduced	GSH in tissue used
	gm.	per cent	mg.
Liver brei.....	0.8	12.1	2.76
“ “ with iodoacetate.....	0.8	None	
“ “ heated.....	0.8	43.5	
	ml.		
Blood diluted 1:1 with isotonic phosphate buffer.....	2	7.6	1.23
Same, with iodoacetate.....	2	None	
“ heated.....	2	20.0	
Red blood cells suspended in an equal volume of saline-phosphate.	2	11.0	2.09
Same, heated.....	2	40.9	
Plasma.....	4	None	None
“ heated.....	4	“	
	gm.		
Muscle brei.....	1	3.6	0.45
“ “ with iodoacetate.....	1	None	
“ “ heated.....	1	5.8	
Small intestine, ground.....	0.8	6.2	1.36
Same, with iodoacetate.....	0.8	None	

that glutathione and fixed —SH compounds were responsible for the reduction.

*Effect of Incubation on Glutathione Content of Liver*—We were unable to find a strictly quantitative relationship for the disappearance of 2 moles of GSH per mole of dehydroascorbic acid reduced, because the reducing power of liver brei as measured by iodine titration increases rapidly upon standing.

Hopkins and Elliot (28) observed an increase in glutathione content during the incubation of chopped liver from well fed rabbits and cats. They attributed this to the reduction of oxidized glutathione by the tissue. Analyses carried out on guinea pig livers require a different interpretation, however. The liver was ground as usual in a frozen condition (with dry ice) and

TABLE V

*Increase of Total and Reduced Glutathione in Liver Brei upon Standing at Room Temperature*

The results are expressed in mg. of glutathione per gm. of liver.

Time, min. . . . .	0	10	20	30	40	50	60
Reduced glutathione . . . . .	2.86	3.09	3.36	3.80	4.43	4.24	4.34
Total " . . . . .	2.86	3.22	3.43	3.83	4.34	4.48	4.52
Ratio, reduced to total glutathione . . . . .	1.0	0.96	0.98	0.99	1.02	0.95	0.96

suspended in M/15 phosphate, pH 7.4. Aliquots were analyzed at once and at intervals while the liver was standing at room temperature. The analyses for total and reduced glutathione by the method of Quensel and Wacholder (29) are shown in Table V. It is evident that the increase in reduced glutathione does not occur at the expense of oxidized glutathione, because it is accompanied by a parallel increase of total glutathione. Our data support the recent suggestion of Rosenbohm (30) that the increase in glutathione under these conditions is due to proteolysis.

*Reduction of Dehydroascorbic Acid by Liver Tissue in Presence of Ascorbic Acid*—The evidence presented here, and by Borsook *et al.*, on the reducing effect of —SH compounds was obtained by adding relatively large amounts of dehydroascorbic acid to tissues.

This does not necessarily prove that small amounts of dehydroascorbic acid would be similarly reduced in the presence of relatively large amounts of ascorbic acid. Yet this is the condition that would prevail in animal tissues during the oxidation of

TABLE VI

*Reduction of Dehydroascorbic Acid by Liver, Heat-Coagulated Liver, and Glutathione in Presence of Ascorbic Acid*

Time 30 minutes; anaerobic, 37.5°, pH 7.4. Total ascorbic + dehydroascorbic acid 2 mg.

Reducing system	Ascorbic acid added	Dehydroascorbic acid added	Added dehydroascorbic acid reduced
	mg.	mg.	per cent
Liver brei, 0.8 gm.	1.80	0.20	0
	0	0.20	44.5
	1.40	0.60	20.0
	0	0.60	32.5
	1.00	1.00	28.0
	0	1.00	28.2
Heat-coagulated liver, 0.8 gm.	1.76	0.24	0
	0	0.24	30.0
	1.64	0.36	2.8
	0	0.36	28.4
	1.52	0.48	16.7
	0	0.48	26.2
Glutathione, 3 mg.	1.76	0.24	4.2
	0	0.24	34.7
	1.64	0.36	16.6
	0	0.36	30.5
	1.52	0.48	18.4
	0	0.48	30.8
	1.40	0.60	35.0
	0	0.60	30.0
	1.00	1.00	28.0
	0	1.00	26.6
	0.60	1.40	28.8
	0	1.40	25.0

ascorbic acid. Information concerning this point is given in Table VI.

It is evident that the presence of ascorbic acid greatly depresses the reduction of dehydroascorbic acid. When the absolute amount of dehydroascorbic acid is decreased, the reduction be-

comes more complete, but when the ratio of ascorbic to dehydroascorbic acid is increased, reduction is less complete. When the excess of ascorbic acid over dehydroascorbic acid is about 3-fold or less, the inhibiting effect of ascorbic acid is not noticeable. Analytical limitations prevent a study of the reduction of dehydroascorbic acid in the presence of 50- to 100-fold excess of ascorbic acid. The results given in Table VI suggest, however, that under those conditions glutathione and other  $\text{—SH}$  compounds would have practically no reducing action on dehydroascorbic acid *in vitro*.

No evidence has been found in any of our experiments to indicate that enzymes are involved in the reduction of dehydroascorbic acid when added to minced tissues. Liver brei poisoned with iodoacetate was unable to reduce dehydroascorbic acid anaerobically in the presence of succinate. Dehydroascorbic acid therefore does not serve as a hydrogen acceptor for the activated hydrogen of the succinate-fumarate system.

#### DISCUSSION

The demonstration (a) that dehydroascorbic acid is antiscorbutically active (31, 32), (b) that its administration leads to increased urinary excretion of ascorbic acid in man (7, 4), and (c) that storage of ascorbic acid occurs in guinea pig tissues (8) after administering dehydroascorbic acid, has established the fact that the animal organism can reduce dehydroascorbic acid. The work of Borsook *et al.* (4) has suggested that glutathione is mainly responsible for the reduction of dehydroascorbic acid added to tissues. Our experiments confirm their work and show further that acid-insoluble  $\text{—SH}$  compounds have a similar effect. Blocking of  $\text{—SH}$  compounds in tissues abolishes their reducing capacity for dehydroascorbic acid, indicating that they are the only or at least the most important factors in the reduction.

If  $\text{—SH}$  compounds are responsible for the maintenance of ascorbic acid (in the reduced state) in tissues, it should be possible to accelerate the disappearance of added ascorbic acid by eliminating the  $\text{—SH}$  groups by the addition of iodoacetate or arsenite. Stotz *et al.* (15) have found, however, that the rate of disappearance of added ascorbic acid in liver brei is not significantly affected by these reagents. Similar doubt concerning the

relation of glutathione to the disappearance of added ascorbic acid in chopped liver tissue has been expressed by Hopkins and Morgan (3). The apparent discrepancies can be reconciled on the basis of our observation (Table VI) that glutathione, liver brei, and fixed —SH of heat-coagulated liver exert little or no reducing effect on dehydroascorbic acid in the presence of a large excess of ascorbic acid. Hopkins and Morgan (3) have pointed out that the addition of glutathione to liver tissue can prevent the oxidation of ascorbic acid. Since the liver contains catalysts that promote the oxidation of ascorbic acid (15), the rate of disappearance of ascorbic acid should depend in part upon the effectiveness of the reducing systems counterbalancing the oxidation. This in turn is conditioned by the relative concentration of the reactants in the system:  $\text{dehydroascorbic acid} + 2 \text{GSH} \rightleftharpoons \text{ascorbic acid} + \text{GSSG}$ . The ratios of these reactants may vary considerably in different tissues, as in the adrenals compared with liver.

Our observation that the reaction is markedly conditioned by the concentration of the reactants and products of the reaction does not exclude the formation of a complex between dehydroascorbic acid and glutathione (4), analogous to that reported for methylglyoxal (33).

#### SUMMARY

1. Dehydroascorbic acid is rapidly reduced when added to suspensions of liver, muscle, small intestine, blood, and erythrocytes, but it is not reduced at an appreciable rate by blood serum (heated or unheated).

2. Heat-coagulated liver, free from glutathione, also reduces dehydroascorbic acid rapidly.

3. Treatment of the tissues with iodoacetate, arsenite, or alloxan inhibits the reduction completely.

4. Glutathione and fixed —SH compounds of the tissues are responsible for the reduction of added dehydroascorbic acid. There was no evidence for enzymic reduction of dehydroascorbic acid.

5. In the presence of a large excess of ascorbic acid, tissues do not reduce dehydroascorbic acid at an appreciable rate.

6. The reduction of dehydroascorbic acid by animal tissues



depends greatly upon the concentration of the components of the system: dehydroascorbic acid + 2 —SH  $\rightleftharpoons$  ascorbic acid +

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## THE OXIDATION OF ASCORBIC ACID IN THE PRESENCE OF GUINEA PIG LIVER\*

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The theory that ascorbic acid may serve as an intermediate or hydrogen transfer agent in tissue respiration has been formulated from several inconclusive types of evidence, although the reversible nature of the oxidation of ascorbic acid to dehydroascorbic acid *in vitro* has been clearly established (1, 2). In the preceding paper (3) special consideration has been given to the tissue agencies that might be responsible for the reduction of dehydroascorbic acid to ascorbic acid.

The literature concerning tissue oxidation of vitamin C includes the following types of evidence: (a) the existence of a specific oxidizing enzyme in plants (4-7); (b) an increased oxygen consumption rate upon adding ascorbic acid to scorbutic liver and muscle *in vitro* (8); (c) certain relationships (1, 6) to a generally accepted respiratory catalyst (9), glutathione; (d) and an observation that "hexuronic" (ascorbic) acid slowly reduced cytochrome (10).

No specific "oxidase" has been observed in animal tissue, however, and a recent paper from this laboratory has shown that the so called ascorbic acid oxidase (in plants) consists chiefly of organically combined copper (11). Further observations showed that the apparent increased oxygen consumption upon adding ascorbic acid to scorbutic tissue was only equivalent to the loss of vitamin during the experiment (12). Two of the arguments

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Contribution No. 349 from the Department of Chemistry, University of Pittsburgh.

proposed in favor of a respiratory function for vitamin C have thus been weakened.

There have been several reports concerning the ability of animal tissue to "protect" the vitamin against aerobic oxidation (12-15), but few papers have dealt with the nature of the agents involved in such reactions.

#### EXPERIMENTAL

The experiments were carried out with the Warburg apparatus, both the Erlenmeyer type and square vessels being used. Unless otherwise stated, the vessels were air-filled and contained 400 mg. of liver as brei and  $m/15$  phosphate buffer (pH 7.3), total volume, 3.3 ml. Normal guinea pigs on the Sherman basal diet (16) with a vitamin C supplement of 2 mg. per day were used in all the experiments reported. The brei was prepared by thorough grinding of the liver with phosphate buffer, so that the concentration was 200 mg. of liver (wet) per ml. The added ascorbic acid<sup>1</sup> (2 mg. in 0.3 ml.) was neutralized with sodium bicarbonate and phosphate buffer. One flask was always "sacrificed" at zero time to determine the initial quantity of ascorbic acid present. At the appropriate time, the contents of each flask were quantitatively washed into a 15 ml. centrifuge tube containing 5 ml. of 10 per cent  $CCl_3COOH$  + 2 per cent  $HPO_3$  solution. An aliquot of the filtrate was titrated with standardized 2,6-dichlorobenzene indophenol. Added ascorbic acid was recovered quantitatively by this procedure.

Indophenol oxidase activity in the oxidation of *p*-phenylenediamine and the activity of "isolated" indophenol oxidase-cytochrome toward ascorbic acid were measured manometrically. Very close agreement was obtained with successive flasks having identical contents.

#### *Oxidation in Presence of Liver Brei*

For economy of space Fig. 1 includes data obtained with varying quantities of both washed and unwashed liver brei and data on the effect of sodium diethyldithiocarbamate, a copper inhibitor. The following points may be noted: (a) In the presence of

<sup>1</sup> We are indebted to Charles Pfizer and Company for a generous supply of ascorbic acid.

a copper inhibitor (Curve B) there was a regular increase in the extent of oxidation with increasing quantities of tissue, but this was obscured in the absence of the inhibitor (Curve A) by the effect of copper in the medium. The first effect of increasing the amount of tissue was to block copper catalysis. (b) With quantities of liver greater than 200 mg., the extent of oxidation increased regularly with increasing weight of tissue, irrespective of washing or the presence of a copper inhibitor. (c) Liver brei

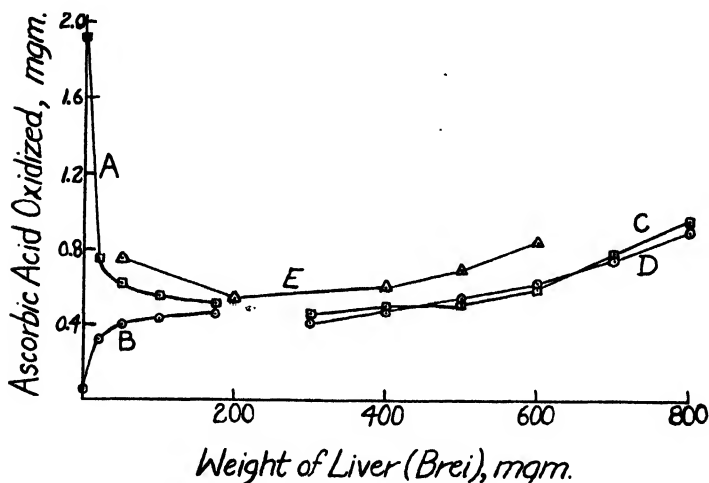


FIG. 1. Aerobic oxidation of ascorbic acid by liver brei.  $T$   $37^{\circ}$ , pH 7.3,  $M/15$   $PO_4$ , total ascorbic acid 2.04 mg., time 2 hours. Curves B and D, unwashed brei + diethyldithiocarbamate (0.006  $M$ ); Curves A and C, unwashed brei; Curve E, washed brei. Curves A and C were obtained under the same experimental conditions, but with different livers; Curves B and D are similarly related. All points on Curve E were established with samples from the same livers.

that had been washed exhibited the same characteristics (Curve E) as the original tissue. (d) Since Curves C and D were obtained with the same samples of liver, they are strictly comparable, and show clearly that diethyldithiocarbamate had no effect on the liver agency responsible for the oxidation of the vitamin.<sup>2</sup>

<sup>2</sup> Although diethyldithiocarbamate interferes with the indophenol titration when added directly to the titration flask, its presence was without effect upon the filtrate after protein was precipitated with  $CCl_3COOH + HPO_4$ .

This is the first evidence that copper or copper proteinate is not responsible for the catalytic effect of liver brei. (e) Curves A and B are also strictly comparable, and demonstrate that in the presence of less than 100 mg. of tissue the copper contamination was not completely bound by the protein. (f) Curve E represents different livers than do Curves C and D, hence the

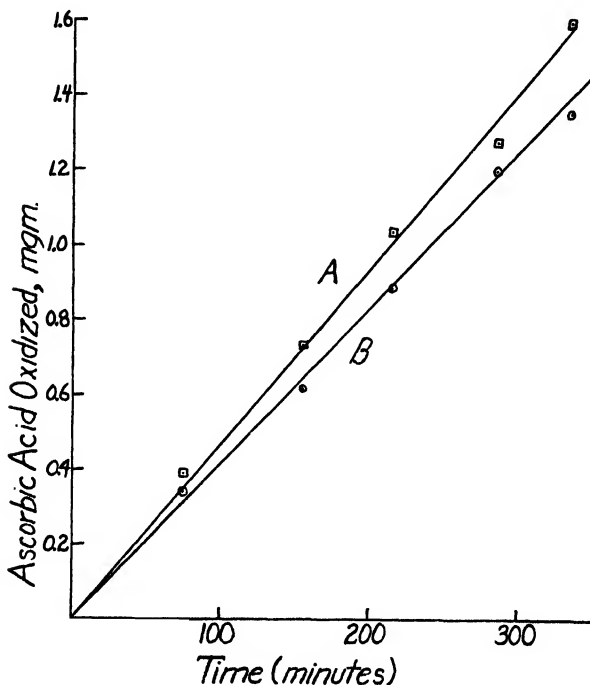


FIG. 2. Time course of the aerobic oxidation of ascorbic acid by liver brei. pH 7.3,  $M/15$   $PO_4$ ,  $T$   $37^\circ$ , 400 mg. of liver (brei), total ascorbic acid 2.05 mg. Curve A, unwashed brei; Curve B, washed brei.

curves are not strictly comparable, but under the conditions of the experiments described, only about 0.5 mg. of ascorbic acid was oxidized in the presence of 400 mg. of liver brei. This is a relatively slow rate (0.4 to 0.6 mg. in 2 hours) when considered in relation to the total respiration capacity of liver tissue.

*Time Course of Oxidation*—Although different livers caused somewhat different rates of oxidation, the course of the oxidation

was essentially linear in all cases for at least 60 per cent of the oxidation. Typical results are recorded in Fig. 2 for washed and unwashed brei, from different livers.

*Effect of pH*—The rate of oxidation was sensitive to pH changes, increasing as the pH was raised through pH 7.3, as illustrated in Table I for both washed and unwashed brei.

*Effect of O<sub>2</sub> Tension*—The rate of oxidation was also dependent on the oxygen tension. When the flasks were filled with identical reactants but with varying gas mixtures, the oxidation rate was decreased 16 per cent by changing from 100 per cent O<sub>2</sub> to 50 per cent O<sub>2</sub> plus 50 per cent N<sub>2</sub>, and 35 per cent by 10 per cent O<sub>2</sub> plus 90 per cent N<sub>2</sub>.

*Effect of Heat*—Washed liver brei lost 80 to 85 per cent of its activity when held at 100° for 5 minutes, indicative of enzyme

TABLE I

*Influence of pH on Aerobic Oxidation of Ascorbic Acid in Presence of Liver Brei*

*T* 37°, *M*/15 PO<sub>4</sub> buffer, 200 mg. of liver, time 3 hours, total ascorbic acid 2.02 mg.

pH.....	6.04	6.42	6.60	6.85	7.01	7.33
	mg.	mg.	mg.	mg.	mg.	mg.
Ascorbic acid oxidized.....	0.54	0.61	0.65	0.72	0.79	0.90

inactivation. Washing removes blood pigments and substances essential to the functioning of the peroxidase-quinone system (17), but washing alone did not cause an appreciable loss in activity. In the presence of hemolyzed blood, pyridine-KCNS and 8-hydroxyquinoline both caused an increased rate of oxidation, accompanied by the appearance of a green pigment. The latter suggested the functioning of a new hemochromogen observed by Lemberg (18), and also provided an explanation for the fact that heating unwashed brei did not result in a decreased rate of oxidation.

#### *Inhibitors of Oxidation*

With *washed* tissue, it was found that the copper inhibitors diethyldithiocarbamate, 8-hydroxyquinoline, and pyridine-KCNS

(11) had no effect on the rate of oxidation, while cyanide, carbon monoxide, and sodium azide (at pH 6.4) markedly inhibited the reaction. Carbon monoxide and cyanide are copper inhibitors, but sodium azide was found to have no effect on copper catalysis. Keilin has previously used the above second group of inhibitors for blocking the action of indophenol oxidase. These facts led to the belief that the indophenol oxidase-cytochrome system was responsible for the oxidation observed in the tissue brei. The

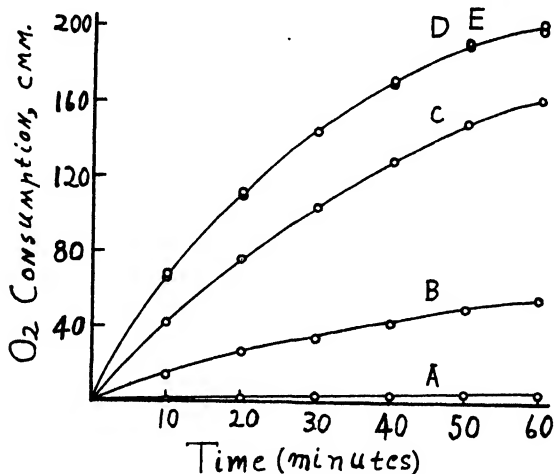


FIG. 3. Oxidation of ascorbic acid by the indophenol oxidase-cytochrome system.  $T$  37°, pH 7.3,  $m/15$   $PO_4$ , total ascorbic acid 0.02 mm. Curve A, cytochrome (13 micrograms of Fe) + diethyldithiocarbamate (0.006 M); Curve B, indophenol oxidase + diethyldithiocarbamate (0.006 M); Curve C, indophenol oxidase + 6.5 micrograms of Fe (as cytochrome); Curve D, indophenol oxidase + 13.0 micrograms of Fe (as cytochrome); Curve E, indophenol oxidase + 13.0 micrograms of Fe (as cytochrome) + diethyldithiocarbamate (0.006 M).

results of Barron *et al.* (15) with hemochromogens are also of interest, however.

#### *Oxidation of Ascorbic Acid by Indophenol Oxidase-Cytochrome System*

Indophenol oxidase was prepared from beef heart (19), and cytochrome C was prepared by a combination of the methods of Theorell (20) and of Keilin and Hartree (21). The cytochrome,

after dialysis against dilute ammonia, was found to contain 0.31 per cent Fe (90 per cent pure), estimated by the method of Lintzel (22).

The effect of these preparations on ascorbic acid oxidation is summarized in Fig. 3. The following points were observed: (a) Cytochrome C alone was unable to catalyze the aerobic oxidation of ascorbic acid. The apparent activity of the preparation was entirely blocked by a copper inhibitor (diethyldithiocarbamate). (b) The indophenol oxidase preparation catalyzed the oxidation of ascorbic acid independently of its copper content. (c) The addition of cytochrome C to indophenol oxidase caused a marked increase in the rate of oxidation of ascorbic acid.

These experiments are similar to those of Keilin (23) in which cysteine was used as the oxidizable substrate. The indophenol oxidase preparation undoubtedly contained some cytochrome, but attempts to remove it by kieselguhr or cellophane adsorption resulted in only small decreases in catalytic activity. It was of interest to note also that guinea pig brain, a tissue rich in indophenol oxidase (manometric *p*-phenylenediamine oxidation), was nearly devoid of oxidizing power for ascorbic acid. The addition of cytochrome, however, induced a rapid oxidation, as it did when added to liver brei. This effect too was independent of copper.

Omitting exact details, we found that the isolated indophenol oxidase-cytochrome system closely resembled the tissue brei in its reaction to diethyldithiocarbamate, cyanide, carbon monoxide, sodium azide, pH, and oxygen tension.

Since, *in vivo*, there is a very low concentration of ascorbic acid to serve as substrate for an oxidizing system, it was of interest to examine the velocity-substrate relations with this isolated oxidizing system. Manometrically measured oxidation rates corresponded to nearly straight lines, from which the oxidation rates recorded in Table II were taken. The fact that the velocity of oxidation was still increasing in this relatively high range of substrate concentration suggests that at physiological concentrations the oxidation of ascorbic acid by this system would be very slow.

Since the evidence pointed to the indophenol oxidase-cytochrome system as the major factor in the tissue oxidation of the vitamin, it was of interest to compare cyanide inhibition of ascorbic



acid oxidation with its effect upon *p*-phenylenediamine oxidation as recorded in Table III. The results show that indophenol oxidase may be partially poisoned without causing an equivalent inhibition of the oxidation of ascorbic acid. Since the oxidation of ascorbic acid can be completely poisoned by cyanide at higher concentration (0.15 mM NaCN), it appears that cytochrome rather than indophenol oxidase was the limiting factor in the oxidation. This view is substantiated by the fact that indophenol oxidase, when added to liver brei, produced only slight increases

TABLE II

*Velocity-Substrate Relations in Aerobic Oxidation of Ascorbic Acid by Indophenol Oxidase-Cytochrome*

*T* 37°, pH 7.3, *m*/15 PO<sub>4</sub>, 3.25 micrograms of cytochrome Fe, 0.5 ml. of indophenol oxidase preparation. The results are expressed in c.mm. of O<sub>2</sub> in 30 minutes.

Ascorbic acid, <i>mM</i> .....	0.0066	0.0083	0.0100	0.0133	0.0166	0.0200
Velocity.....	32	39	44	52	60	63

TABLE III

*Comparison of Cyanide Inhibition of Ascorbic Acid Oxidation and *p*-Phenylenediamine Oxidation in Presence of Liver Brei*

pH 7.3, *m*/15 PO<sub>4</sub>, 400 mg. of liver (brei), 0.02 mM of ascorbic acid, 0.3 ml. of 1 per cent *p*-phenylenediamine. The inhibitions are expressed in per cent.

NaCN, <i>mM</i> .....	0.005	0.015	0.030	0.050
Inhibition of <i>p</i> -phenylenediamine oxidation...	21	57	71	81
“ “ ascorbic acid oxidation.....	6	19	32	57

in the rate of oxidation, whereas very small additions of cytochrome caused complete and rapid oxidation of the vitamin.

#### *Relations with Glutathione*

Thus far we have considered only the over-all disappearance of the vitamin in the presence of liver brei, without reference to the possible effect of glutathione and other reducing systems that might cause reduction of the acid from its reversibly oxidized form. If concurrent reduction were the reason for the apparent

slow oxidation, as measured by titration, destruction of the glutathione or dehydrogenase systems should cause an apparent increase in the rate of oxidation. The following evidence is contrary to such an interpretation: (a) Ethyl urethane (1.2 per cent) had no effect on the rate of oxidation, indicating that dehydrogenases were not involved. (b) Reduced glutathione disappeared from the brei at essentially the same rate whether or not ascorbic acid was added (1.9 mg. per 0.75 gm. of brei). (c) Incubation of tissue with iodoacetate or arsenite (0.01 M) did not affect the apparent rate of oxidation, although these inhibitors have been shown by Schultze *et al.* (3) completely to block the reduction of dehydroascorbic acid by glutathione. (d) When dehydroascorbic acid is accompanied by an excess of ascorbic acid as it is in animal tissues, little or no reduction by sulfhydryl compounds occurs.

We have repeated and confirmed the experiments of Hopkins and Morgan (6) on the relations of ascorbic acid and glutathione in liver brei. However, when both glutathione (50 mg.) and ascorbic acid (1.2 mg.) were added to liver brei (400 mg.), so that the ratio of the two was essentially that found *in vivo*, the glutathione fully "protected" the ascorbic acid from oxidation.<sup>3</sup>

In studying the disappearance of the vitamin naturally present in liver brei, no difference could be observed between brei alone and that incubated with arsenite. This was true at both high and low oxygen tensions (50 and 10 per cent O<sub>2</sub>). Thus under conditions that were physiological in the sense of absolute and relative amounts of the reactants, glutathione apparently exercised no effect on the disappearance of ascorbic acid.

In view of the hypothesis (1) that certain dehydrogenase systems might react with oxygen through the chain substrate-dehydrogenase-glutathione-ascorbic acid-cytochrome-indophenol oxidase, we studied the reaction of glutathione and ascorbic acid with the cytochrome-indophenol oxidase system. The results may be summarized as follows: (a) Glutathione, like ascorbic acid, was aerobically oxidized in the presence of copper, and the oxidation was completely inhibited by diethyldithiocarbamate. (b) Glutathione did not require the presence of ascorbic acid to be oxidized rapidly by the cytochrome-indophenol oxidase system.

<sup>3</sup> The quantities of glutathione used by us did not interfere with a rapid indophenol titration of ascorbic acid.

(c) When both glutathione (0.01 mM) and ascorbic acid (0.003 mM) were present with indophenol oxidase-cytochrome, the ascorbic acid disappeared more rapidly than the glutathione. This does not suggest a relation between ascorbic acid and glutathione in animal tissues, such as was found in plants by Hopkins and Morgan (6).

The effects of oxidation inhibitors were essentially the same with tissue slices and with liver brei.

#### DISCUSSION

In discussing the possibility of ascorbic acid being an important oxidation-reduction mediator, it seems fair to compare it to such accepted mediators as cytochrome or "yellow enzyme." Both of these substances are *rapidly* reduced and oxidized by physiological respiratory systems.

The preceding paper and this one have cast some doubt on the normal rôle of glutathione in reducing small amounts of dehydro-ascorbic acid in the presence of an excess of ascorbic acid, to complete a major respiratory cycle.

Although we have shown that rapid oxidation of the vitamin may occur through the isolated indophenol oxidase-cytochrome system, the observed oxidation in liver brei was very slow. When we consider the great decrease in oxidation velocity that is due to low oxygen tension and low substrate concentration in the cell, it appears unlikely that a large part of the total respiratory exchange would pass through the medium of ascorbic acid. However, we must consider the recent arguments of Theorell (24) and Green and Richter (25), that the activity of indophenol oxidase-cytochrome is greater *in vivo* than *in vitro*.

At the present time there is considerable evidence against, and little evidence for, the function of ascorbic acid as a major respiratory agent in animal tissues, at least in the sense of being comparable to cytochrome or "yellow enzyme." The possible function of the vitamin in plant respiration is not considered here, however, nor do we wish to imply that ascorbic acid does not play an important rôle in the metabolism of animals.

#### SUMMARY

1. Guinea pig liver brei brought about a slow aerobic oxidation of added vitamin C.

2. The characteristics of the reaction and the effects of inhibitors indicated that the indophenol oxidase-cytochrome system was chiefly responsible for the oxidation. Cytochrome was the limiting factor in the catalytic activity of both liver and brain.

3. A rapid oxidation of ascorbic acid was observed when both indophenol oxidase and cytochrome, prepared from beef heart, were used together.

4. No evidence was obtained to indicate the presence of either a specific oxidase or active tissue copper, in relation to the oxidation of ascorbic acid added to liver.

5. The glutathione naturally present in liver brei was unable to "protect" added ascorbic acid from oxidation.

6. Ascorbic acid was not an essential intermediate in the reaction of glutathione with oxygen, either through the indophenol oxidase-cytochrome system or in the presence of copper.

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## THE ERGOT ALKALOIDS

### XIII. THE PRECURSORS OF PYRUVIC AND ISOBUTYRYLFORMIC ACIDS

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(Received for publication, October 28, 1937)

In previous studies<sup>1</sup> we have shown that each of the pair of alkaloids, ergotoxine-ergotinine, is composed of lysergic acid (or an isomer), ammonia, isobutyrylformic acid, *d*-proline, and *l*-phenylalanine combined in amide linkages. The pair ergotamine-ergotaminine is similarly constructed, with the exception that pyruvic acid replaces the isobutyrylformic acid in the former case. Thus, the addition of the five components of ergotinine and ergotoxine with cleavage of 4 moles of H<sub>2</sub>O would give C<sub>35</sub>H<sub>39</sub>O<sub>5</sub>N<sub>5</sub>, which is the accepted formula of ergotinine. In the case of the ergotamine-ergotaminine pair, addition of its five components with cleavage of 4 moles of H<sub>2</sub>O would give the accepted formula C<sub>33</sub>H<sub>35</sub>O<sub>5</sub>N<sub>5</sub>.

When these alkaloids are treated with alcoholic alkali (Smith and Timmis<sup>2</sup>), the amide of lysergic acid (ergine) is formed. And since this amide so readily yields lysergic acid<sup>3</sup> and ammonia with aqueous alkali, it is obvious that the so called ammonia constituent of these alkaloids must be identical with that which emerges as lysergamide.

This at once limits the orders which can be considered in regard to the arrangement of the components of the alkaloids. Further, the fact that these alkaloids give only monoacid salts due to the

<sup>1</sup> Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, **110**, 521 (1935); *J. Org. Chem.*, **1**, 245 (1936).

<sup>2</sup> Smith, S., and Timmis, G. M., *J. Chem. Soc.*, 763, 1543 (1932).

<sup>3</sup> Smith, S., and Timmis, G. M., *J. Chem. Soc.*, 674 (1934); *Nature*, **133**, 579 (1934). Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, **106**, 393 (1934).

basic  $\text{CH}_2\text{N}$  group of lysergic acid also excludes from consideration a terminal phenylalanyl or prolyl residue with unacylated amino or pyrrolidyl groups, and more recently we have found that ergotinine and ergotamine give no amino nitrogen in the Van Slyke apparatus. It could be concluded from these facts that the alkaloid components are arranged in such a way that either of the amino acids phenylalanine or proline forms a diacyl amide with lysergic acid, and isobutyrylformic acid or pyruvic acid is situated at the end of the chain.

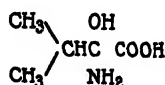
Such an arrangement, however, which assumes a diacyl amide linkage appeared very improbable and we have attempted to check another possibility already suggested by us on a former occasion;<sup>4</sup> *viz.*, that isobutyrylformic acid and also pyruvic acid do not occur as such in these alkaloids but are formed by decomposition of a precursor. Direct evidence of this has now been found.

When ergotinine is hydrogenated in acetic acid with platinum oxide catalyst, a prompt absorption of hydrogen required for saturation of the double bond of lysergic acid occurs and then continues more gradually.<sup>5</sup> The reason for the excessive absorption has not been definitely determined but it is possibly due to continued reduction of the indole nucleus. After several days the total absorption amounted to approximately 3 moles. When the resulting material was hydrolyzed by alkali, isobutyrylformic acid was isolated in approximately the same yield as previously obtained by alkaline hydrolysis of ergotinine itself and was identified as the phenylhydrazone. No  $\alpha$ -hydroxyisovaleric acid could be detected. As a control, isobutyrylformic acid itself was hydrogenated under similar conditions and was found to be reduced readily and quantitatively to  $\alpha$ -hydroxyisovaleric acid. It is obvious, therefore, that either isobutyrylformic acid, when combined as such in the alkaloid, cannot be catalytically reduced or—what is far more probable—it is formed from a precursor during hydrolysis. A study of ergotamine confirmed the latter conclusion. In this case the problem was made simpler by the characteristic color reaction given by pyruvic acid with nitroprusside. As

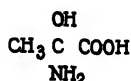
<sup>4</sup> Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, **110**, 523 (1935).

<sup>5</sup> Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, **113**, 767 (1936); **115**, 227 (1936).

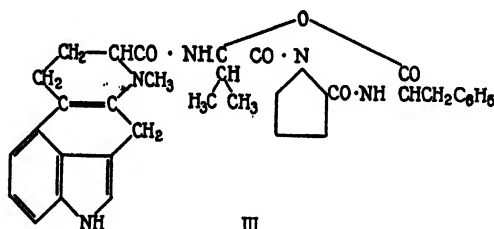
previously reported, when ergotamine or its isomer is hydrolyzed by alkali the resulting mixture gives a strong positive test for pyruvic acid. Neither of these alkaloids, however, gives such a reaction before hydrolysis. When ergotamine was hydrogenated as in the case of ergotinine, the resulting hydrogenated product gave no test directly with nitroprusside; but after alkaline hydrolysis, a test was obtained indistinguishable in intensity from that given by the hydrolyzed unhydrogenated alkaloid.



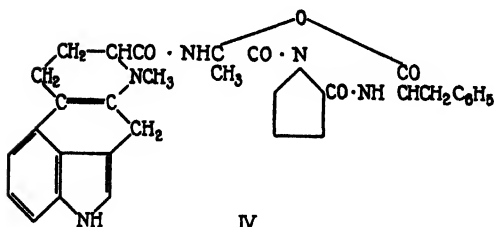
I



II



III



IV

The most satisfactory explanation for these observations is that the precursor of isobutyrylformic acid in the pair ergotinine-ergotamine is  $\alpha$ -hydroxyvaline (I) and that of pyruvic acid in ergotamine-ergotaminine is  $\alpha$ -hydroxyalanine (II). Such substances in the free state should be unstable and decompose readily



into ammonia and the keto acids. When the amino group, however, is protected by acylation as it occurs in the alkaloid, greater stability should be produced.

Since lysergamide (ergine) is produced from the alkaloids with alcoholic alkali, it is obvious that lysergic acid must be directly attached to the amino groups of these  $\alpha, \alpha'$ -hydroxyamino acids which are then in turn joined in peptide linkage with either of the remaining amino acids proline and phenylalanine. Finally, the carboxyl group of the terminal amino acid must form lactones with the hydroxyl groups of hydroxyvaline and hydroxyalanine. The formulas of the ergotinine-ergotoxine pair would be as represented in (III), and of the ergotamine-ergotaminine pair as given in (IV). The position of the carboxyl group of lysergic acid is still tentative, as well as the order in which proline and phenylalanine occur. It appears probable, however, that phenylalanine will be found to be the terminal amino acid in the alkaloids under discussion, since the replacement of phenylalanine by leucine would change ergotamine-ergotaminine into the pair of alkaloids recently shown to make up Küssner's ergoclavine.<sup>6</sup> This question is still a subject of investigation. Also only one of the three possible positions of the double bond of lysergic acid is given in the formulas which may function in the isomerism within each alkaloid pair.

Such formulas now appear to explain satisfactorily the formulations of these alkaloids, their monobasic character, and the origin of the keto acids, ergine and ammonia. Ergine (or its isomer) thus is an artifact and does not occur as such in the alkaloid molecule. There is a possibility that certain influences may affect the stability of the lactone groups of these alkaloids. This may explain the apparent greater acidity of ergotoxine and some of the analytical results obtained with its derivatives. The hydroxy acid would have the formula  $C_{35}H_{41}O_6N_5$  which has been the one commonly accepted for ergotoxine. However, this point is now under further investigation and it is possible that the so called  $\psi$ -ergotinine of Smith and Timmis<sup>7</sup> will have to be interpreted in

<sup>6</sup> Küssner, W., *E. Merck's Jahresber.*, **47**, 5 (1933); *Z. angew. Chem.*, **50**, 34 (1937). Jacobs, W. A., and Craig, L. C., *J. Org. Chem.*, **1**, 245 (1936). Smith, S., and Timmis, G. M., *J. Chem. Soc.*, 396 (1937).

<sup>7</sup> Smith, S., and Timmis, G. M., *J. Chem. Soc.*, 1888 (1931).

this connection and may prove to be the hydroxy acid of the lactone, ergotinine.

#### EXPERIMENTAL

0.5 gm. of ergotinine was hydrogenated in 3 cc. of acetic acid with 0.05 gm. of Adams and Shriner's platinum oxide catalyst under 2 atmospheres pressure. Approximately 3 moles of  $H_2$  were absorbed. The filtered solution was concentrated to dryness *in vacuo*. The residue was treated with a solution of 1.4 gm. of KOH in 10 cc. of 50 per cent methyl alcohol and the mixture was refluxed in a current of  $H_2$  for 30 minutes. After dilution with water and removal of methyl alcohol under reduced pressure the mixture was acidified with  $H_2SO_4$  and extracted with ether. The dried ether solution left an oil on concentration, which was sublimed at 20 mm. in a bath heated up to  $120^\circ$ . 20 mg. of oil were collected. This gave at once a crystalline phenylhydrazone with phenylhydrazine in dilute acetic acid. After recrystallization from dilute alcohol, needles were obtained which melted at  $152^\circ$ .

$C_{11}H_{14}O_2N_2$ . Calculated, C 64.08, H 6.84; found, C 63.96, H 6.88

No evidence of the formation of  $\alpha$ -hydroxyisovaleric acid could be obtained.

35 mg. of isobutyrylformic acid which was obtained as above from ergotinine were dissolved in 2 cc. of alcohol and hydrogenated with 25 mg. of platinum oxide catalyst. In 1 hour hydrogenation stopped after absorption of 1 mole of  $H_2$ . After the material was filtered and concentrated, a syrup was obtained which was first sublimed at 20 mm. in a bath heated to  $100^\circ$ . The sublimate was recrystallized from petroleum ether and yielded 15 mg. of crystals which melted at  $81-82^\circ$ .

$C_5H_{10}O_3$ . Calculated, C 50.81, H 8.55; found, C 50.95, H 8.42



## THE METABOLISM OF CYSTINE AND METHIONINE

### II. THE AVAILABILITY OF *d*- AND *l*-METHIONINE AND THEIR FORMYL DERIVATIVES IN THE PROMOTION OF GROWTH\*

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(Received for publication, October 13, 1937)

In our detailed report (2) of studies showing that *dl*-methionine can replace cystine as an essential dietary supplement for the promotion of growth, we outlined the possible metabolic interrelations<sup>1</sup> of these amino acids together with several outgrowing problems which subsequently have been investigated in various laboratories. One of these problems was to determine whether only one or both of the optically active forms of methionine would promote growth and further whether the corresponding acylated derivatives were physiologically available. These com-

\* A considerable part of the experimental work was done in the Laboratory of Physiological Chemistry, Yale University, and a preliminary note has been published (1).

<sup>1</sup> Inasmuch as the basal diet employed in our investigation contained small amounts of both cystine and methionine, one possible interpretation which we suggested for our results was that only one of the two amino acids, cystine or methionine, is indispensable and that there is at least one metabolic function which can be cared for by either amino acid or by some common metabolite of the two. This seems to explain both our results and the findings of Professor Rose and collaborators (3). They report that, "Diets entirely devoid of cystine, except for the traces which may be furnished by the vitamin supplements, support rapid growth, provided methionine is present. On the other hand, when cystine is furnished in place of methionine, the animals fail to grow, or do so at greatly diminished rates." Further evidence of the importance of methionine has been secured, as we originally suggested, in experiments (Brand, Cahill, and Harris (4)) showing that the administration of methionine to the cystinuric patient increases the excretion of cystine.

## 426 Methionine Isomers and Formyl Compounds

pounds were of special interest from the standpoint of the comparative physiology of the amino acids, inasmuch as previous tests by other investigators had revealed that the unnatural *d*-cystine does not support growth (5, 6), that both *l*- and *d*-tryptophane are available (7, 8), but that acetyl-*d*-tryptophane, unlike the acetyl derivative of the natural *l*-tryptophane, is unavailable for growth (8, 9).

For our experiments, the formyl derivatives of *d*- and *l*-methionine were selected as the acylated forms to be studied. These and the optical antipodes of the amino acid were secured through the resolution of *dl*-methionine. The physiological tests showed (1) that, relative to the influence of spatial configuration and to the effect of blocking the amino group, methionine is similar to tryptophane. We are presenting herewith a more complete account of our experiments.

### EXPERIMENTAL

#### *Preparation of d- and l-Methionine and of Formyl-d- and Formyl-l-Methionine*

*dl*-Methionine was resolved, except for stated modifications, according to Windus and Marvel (10). 25.0 gm. of the synthetic amino acid yielded 26.3 gm. of formyl-*dl*-methionine, all of which was converted to the brucine salts. The less soluble brucine salt, that of formyl-*d*-methionine, after recrystallization from absolute ethanol amounted to 41.5 gm. and melted at 143–144°<sup>2</sup> following slight initial softening at 131°. The more soluble salt was secured with some difficulty. Progressive concentration of the filtrate from the brucine salt of formyl-*d*-methionine yielded about 1 gm. of an intermediate fraction which was discarded and finally a crop of the more soluble brucine salt of formyl-*l*-methionine. However, the latter was secured only after the volume of solution was reduced sufficiently to cause first the precipitation of a fairly large fraction of free brucine. Inasmuch as it was obvious that the considerable excess of brucine employed was interfering with the crystallization of the brucine salt, the free brucine was removed. The ethanol solution was evaporated to dryness and the residue triturated with ice-cold water. The insoluble

<sup>2</sup> All melting points are corrected.

hydrated brucine was filtered off and washed with more cold water. The combined water filtrates were evaporated and thoroughly dried by repeated distillation of added benzene. The residue was finally crystallized from a minimal amount of absolute ethanol. The total yield of the more soluble brucine salt amounted to 40.7 gm., which melted at 195–198°. The quantity of each brucine salt calculated from the formyl-*dl*-methionine employed should be 42.5 gm. The yields, respectively 41.5 and 40.7 gm. (97 per cent of the total possible 85 gm.), and the widely separated and rather sharp melting points (*cf.* (10)) are excellent evidence of a complete separation of the two enantiomorphs.

The formyl-*d*- and *l*-methionines were secured as directed by Windus and Marvel and crystallized from ethyl acetate, although the resolved products, as contrasted to the racemic formyl methionine, are too soluble in this solvent for convenient manipulation. The formyl-*d*- and *l*-methionines yielded respectively the following analyses: nitrogen calculated 7.91; found 8.05 and 8.01 per cent;  $[\alpha]_D^{25} = +12.2^\circ$  and  $-11.0^\circ (\pm 1.0^\circ)$  for 1.7 per cent solutions in water. The formyl-*l*-methionine melted at 98–100°. For the two compounds, Windus and Marvel report the melting point of 99–100° and specific rotations of  $+10.6^\circ$  and  $-10.0^\circ$  respectively. No evidence of free amino acid in our two preparations could be secured by a controlled semiquantitative application of the ninhydrin test.

The *d*- and *l*-methionines were prepared by hydrolyzing the corresponding formyl derivatives with 2.5 N sulfuric acid for 1½ hours on the steam bath. The sulfuric acid was removed by addition of an equivalent amount of barium hydroxide, and the filtrate from the barium sulfate evaporated *in vacuo* to dryness. The residue was crystallized from 65 per cent ethanol. Three crops of each antipode were thus secured, the third crop in each case being impure. Of the original 22.2 gm. of *dl*-methionine employed in the resolution as the formyl compound, 10.3 and 10.7 gm. were recovered as the *d*- and *l*-methionines or their formyl derivatives. These recoveries thus amounted to more than 90 per cent. The nitrogen values for the *d*- and *l*-methionines were found to be respectively 9.23 and 9.30 per cent (calculated 9.39 per cent).  $[\alpha]_D^{25}$  for 2 per cent solutions of the recrystallized substances in water was respectively  $+7.0^\circ$ ,  $+7.2^\circ$ , and  $-7.4^\circ$ ,

## 428 Methionine Isomers and Formyl Compounds

$-7.1^{\circ} (\pm 0.5^{\circ})$ .<sup>3</sup> Windus and Marvel found rotations of  $+8.12^{\circ}$  and  $-7.5^{\circ}$  (also  $-8.1^{\circ}$ ) for the resolved synthetic materials and  $-6.9^{\circ}$  for the amino acid isolated from casein. For the latter type of preparation, Mueller (11) found a rotation of  $-7.2^{\circ}$ .

### *Physiological Tests*

The technique employed in the growth tests was essentially the same as that described in detail in former publications on cystine and methionine (2, 12). Young male albino rats exhibiting a satisfactorily restricted growth on the basal cystine-methionine-deficient diet (Diet B) were selected for experiment. This diet was composed as follows: whole milk powder 12, gelatin 3, Osborne and Mendel's salt mixture (13) 1.5, sodium chloride 1.0, corn-starch 56.9, tryptophane 0.02, Lloyd's reagent adsorbate<sup>4</sup> of vitamin "B" 0.6, and lard 25 per cent. 100 mg. of cod liver oil and 125 mg. of dried yeast were given separately to each animal daily. The amounts of supplements employed for 100 gm. of the basal diet were 149 and 298 mg. (arbitrarily called 1 and 2 equivalents) of the methionine stereoisomers and 177 and 354 mg. (1 and 2 equivalents) of the formyl methionine stereoisomers.

### DISCUSSION

The effect on growth of each compound was tested with three or more animals. Two experiments with each compound are depicted in Figs. 1 and 2. The experiments uniformly demonstrated that both *d*- and *l*-methionine (*cf.* Rose (14)) as well as formyl-*l*-methionine support growth, whereas formyl-*d*-methionine does not. The negative results for the formyl-*d*-methionine were confirmed with an additional preparation synthesized directly from the authenticated *d*-methionine.

Our results with *d*- and *l*-methionine, therefore, are strictly comparable to those previously reported for tryptophane, and likewise are contrary to the idea that only the naturally occurring optical forms of amino acids are physiologically useful to the animal. It has now been shown that both forms of histidine (15),

<sup>3</sup> The authors thank Dr. William Cahill of Cornell University Medical College for measuring the rotations of the methionine isomers.

<sup>4</sup> This concentrate was kindly supplied by Eli Lilly and Company, Indianapolis.

homocystine (16), and phenylalanine (14), although only the natural form of lysine (17, 18), valine, leucine, and isoleucine (14), are utilizable for growth. The evidence that all naturally occurring amino acids possess the same spatial configuration about the  $\alpha$ -carbon atom, and also the usual marked dependence of physiological response upon a definite chemical pattern and indeed upon a particular optical configuration make it seem curious that the animal can utilize the unnatural form of several amino acids. It appeared unlikely that the animal could incorporate the unnatural isomer in its tissue proteins. In fact, the more reasonable explanation that the natural form is synthesized *in vivo* from its optical isomer has recently been experimentally verified for histidine by Conrad and Berg (19).

Such a conversion is accomplished, it seems fair to assume, through oxidative deamination to the corresponding ketonic acid and subsequent asymmetric synthesis to the natural amino acid. Indeed, the unnatural optical forms of some amino acids are known to yield  $\alpha$ -ketonic acids not only in an isolated system such as a tissue slice, (cf. Krebs (20)) but also in the normal organism. It has not yet been demonstrated for methionine, as it has for several amino acids, that the corresponding ketonic acid promotes growth. Nevertheless, the fact that the *dl*- $\alpha$ -hydroxy (21, 22) and the *dl*-N-methyl (23) derivatives of methionine as well as the unnatural form of the amino acid are utilizable seems to speak for processes involving a common metabolic intermediate and, in particular, the corresponding  $\alpha$ -ketonic acid. In this connection, a comparison of methionine and lysine is of interest. Neither the *dl*-hydroxy (18) nor the *dl*-N-methyl (24) derivative nor the unnatural form of the amino acid is available in place of lysine for growth. This may not mean the failure or absence of three entirely separate physiological mechanisms. On the other hand, it is conceivable that these three substances related to natural lysine are converted to the corresponding  $\alpha$ -keto acid, but that the latter, susceptible to side reaction, is diverted from the more usual amination process.

Our results with the formyl methionines show that the introduction of the formyl group prevents the utilization of the unnatural amino acid but not of its enantiomorph. This finding is analogous to the original observation of du Vigneaud, Sealock,



## 430 Methionine Isomers and Formyl Compounds

and Van Etten (8) with respect to acetyl-*d*-tryptophane. If, as appears most probable, the formyl-*l*-methionine is hydrolyzed in the body to yield the free amino acid, the explanation of the

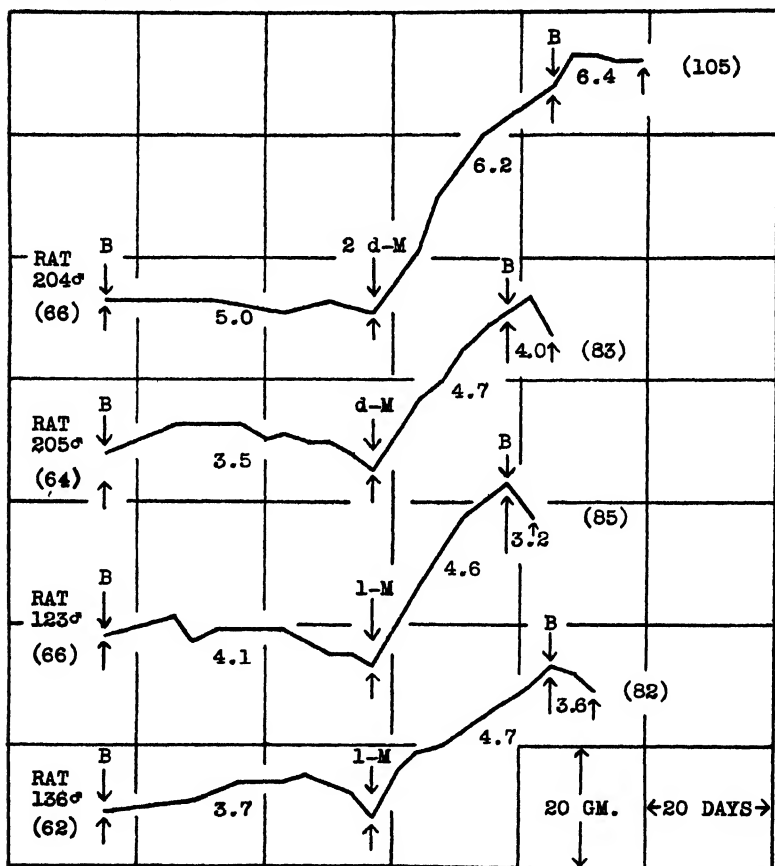


FIG. 1. Growth on the basal deficient Diet B (B), on Diet B supplemented with 1 or 2 equivalents (see text) of *d*-methionine (d-M, 2 d-M) or with 1 equivalent of *l*-methionine (l-M), and finally on Diet B alone. The introduction of each diet is indicated by symbol over a downward arrow. The average daily food consumption in gm. is shown by a number inserted between two upward arrows demarcating the period in question. The initial and final body weights in gm. are printed within parentheses.

different physiological responses elicited by the acylated amino acid stereoisomers must depend on another optically active substance, doubtless a hydrolyzing enzyme. There is abundant

evidence of this type of specificity exhibited by proteolytic enzymes toward variations in the peptide linkage. The observa-

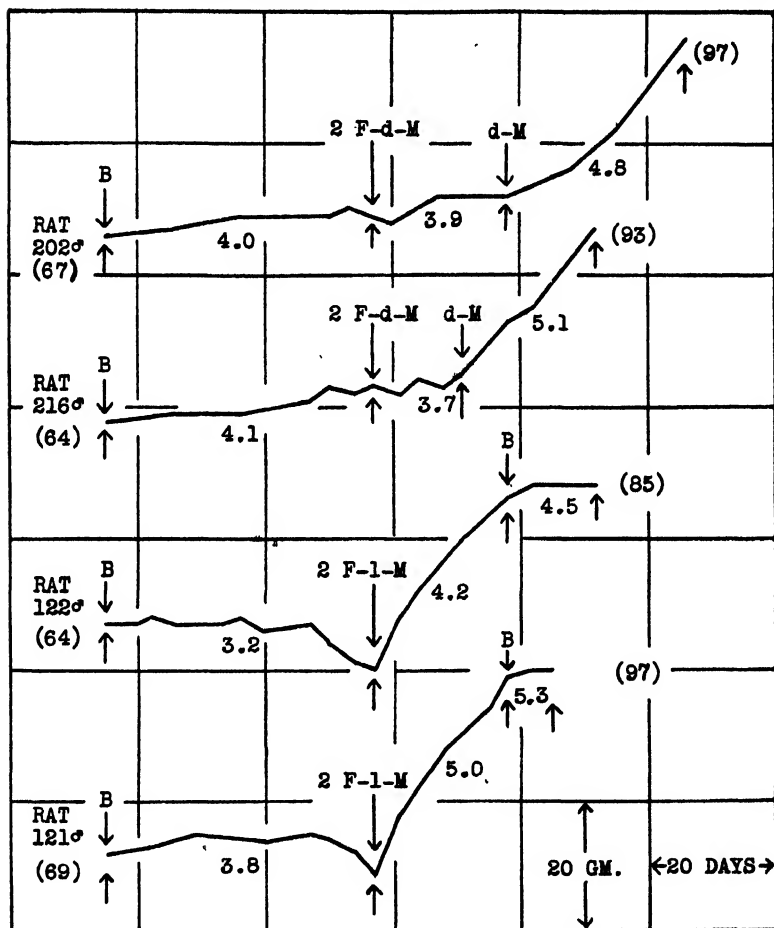


FIG. 2. Growth on the basal deficient Diet B (B), on Diet B supplemented with 2 equivalents of formyl-*d*-methionine (2 F-*d*-M) and then with 1 equivalent of *d*-methionine (d-M); also growth on Diet B (B), on Diet B supplemented with 2 equivalents of formyl-*l*-methionine (2 F-*l*-M), and finally on Diet B alone. Other designations are as defined for Fig. 1.

tions on both tryptophane and methionine reveal that the enzymes available for hydrolyzing the acyl derivatives are more

## 432 Methionine Isomers and Formyl Compounds

limited in their sphere of action than those responsible for the utilization of the stereoisomers of the amino acids themselves.

### SUMMARY

1. Both *d*- and *l*-methionine effectively supplement a ration deficient in cystine and methionine.

2. The formyl derivative of the naturally occurring *l*-methionine is utilized for growth, whereas formyl-*d*-methionine is not.

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## DO ANDROGENS AFFECT THE BLOOD LIPIDS?\*

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There is no conclusive evidence in the literature concerning the relation of androgens to lipid metabolism. Although it is generally considered that castration alters the fat metabolism, this is not a consistent finding. Korenchevsky (1) concluded from a review of the subject as well as his own studies that castration will result in as many thin dogs as fat dogs. Similar results have been noted by one of us (2).

Blinoff (3) found no significant changes in the blood cholesterol values of dogs before and after castration. On the other hand McCullagh, McCullagh, and Hicken (4) observed that injections of androgenic urinary extracts in human patients resulted in a lowering of the blood cholesterol values.

A series of studies (2, 5, 6) on the relationship of androgens to the protein and energy metabolism of dogs has already been reported. In each case there was a decrease in protein metabolism which was accounted for by a lowering of the urinary urea. The other nitrogen constituents of the urine, the non-protein nitrogen and urea of blood, and the energy metabolism were not affected. In one of these studies (androstenedione) (5) blood samples were taken for a simultaneous investigation on blood lipid metabolism (Table I). In addition, the blood lipids of six dogs, 4 to 5 months castrate, were determined. One of these dogs was treated with testosterone oxime and another with testosterone benzoate on the 2 days prior to the second blood sample. The other four dogs were not treated.

\* Supported in part by a grant from the Committee on Scientific Research, American Medical Association.

*Procedure*

*Materials*—The androstenedione was synthesized by Kochakian (5) and dissolved in olive oil in two concentrations, 10 mg. per cc. and 15 mg. per cc. The testosterone oxime<sup>1</sup> was likewise dissolved in olive oil, such that 1 cc. contained 10 mg. The testosterone benzoate<sup>1</sup> was contained in ampules of 2.5 mg. per cc. All solutions were sterile and aseptic procedure was employed during the injections.

TABLE I

*Androstenedione Studies on Dog 1\**

Dog 1, a thin castrate, weighed 15 kilos. The values of the table are given in mg. per cent.

Date	Total cholesterol	Phospholipid	Neutral fat	Total lipid
Apr. 5	151	297	246	694
" 7	151	307	290	748
" 8	190	325	196	711
" 9	161	335	218	714
" 10	141	335	240	716
" 11	151	343	239	733
" 12	198	342	182	722
" 13	179	307	251	737
" 14	174	262	289	725
" 15	180	277	269	726
" 16	156	262	282	700
" 17	131	263	323	717
Average.....	164	305	252	720

\* 60 mg. of androstenedione injected subcutaneously on the mornings of April 6, 7, and 8.

*Dogs*—All the dogs were adult mongrels. Dog 1, castrated in November, 1931, remained thin and very active. Dog 2, castrated in November, 1933, became very fat and sluggish. Dogs 7 to 12 were of varying degrees of fatness and are described in Table II. Dog 6, a normal adult dog, was of approximately the same size as Dog 1.

<sup>1</sup> Testosterone oxime in crystalline form and the testosterone benzoate in sterile ampules of 2.5 mg. per cc. were provided by the Schering Corporation through the courtesy of Dr. Schwenk and Dr. G. Stragnell.

*Diets*—Dogs 1, 2, and 6 were maintained on a constant diet which was fed daily at 5.30 p.m. Dogs 1 and 6 received 75 gm. of beef heart, 60 gm. of cracker meal, 30 gm. of lard, 5 gm. of cod liver oil, 10 gm. of bone ash, and 3 gm. of Wesson's<sup>2</sup> salt mixture. Dog 2 received the same but had 225 gm. of beef heart,

TABLE II

*Relationship of Nutritive State and Castration to Blood Lipid Values*

Dog No.	Nutritive state	Date	Duration of castration		Total cholesterol	Phospholipid	Neutral fat	Total lipid
			yrs.	mos.	mg. per cent	mg. per cent	mg. per cent	mg. per cent
		1938						
1	Thin	Dec. 4	5	1	158	311	252	721
		" 11			147	267	241	655
8	"	" 4		4	168	236	232	636
		" 11*			165	270	232	667
11	Medium fat	" 4		4	143	248	234	625
		" 11			140	255	212	607
10	" "	" 4		4	149	328	254	730
		" 11†			194	322	218	734
9	" "	" 4		4	180	303	232	715
		" 11			164	303	229	696
7	" "	" 4		4	189	283	231	703
		" 11			191	366	258	815
12	Fat	" 4‡		4	364	322	233	919
		" 11‡			333	323	250	906
2	"	" 4	3	1	233	364	331	928
		" 11			222	273	324	819
6§	Thin	Apr. 27	Normal		160	212	322	693

\* 64 mg. of testosterone oxime injected subcutaneously on December 9 and 10.

† 25 mg. of testosterone benzoate injected subcutaneously on December 9 and 10.

‡ The blood cells began to settle almost immediately after withdrawal of the sample.

§ These values are the averages obtained in the experiment with androstenedione while the dog was on the beef heart-cracker meal diet from April 27 to May 7.

80 gm. of cracker meal, and 40 gm. of lard. Dogs 7 to 12 were maintained on a kitchen scrap diet relatively high in carbohydrate. Blood lipid values for Dogs 1 and 2 were also determined while on the latter diet.

<sup>2</sup> Wesson, L. G., *Science*, **75**, 339 (1932).

*Methods*

*Preparation of Blood Samples*<sup>3</sup>—Following a 24 hour fast, 15 to 20 cc. of blood were drawn from the jugular vein (saphenous vein of Dog 1) into a tube containing 0.1 cc. of saturated oxalic acid, previously dried, and immediately centrifuged for 35 minutes.

5 cc. of plasma were drawn off and added slowly with rotation to about 80 cc. of alcohol-ether, both redistilled, according to the method of Bloor (7). After being made up to 100 cc. volume, giving a 1:20 dilution of the original plasma, the extract was filtered and stored in clean bottles stoppered with alcohol-extracted corks.

*Determination of Lipids*<sup>4</sup>—Phospholipid was determined on suitable aliquots of the plasma alcohol-ether extracts by Bloor's method (8) with the modifications as introduced by Boyd (9). The acetone mother liquor and acetone washings after precipitation of the phospholipids were quantitatively transferred to a glass-stoppered 125 cc. Erlenmeyer flask and the solvent evaporated, the last traces being removed completely by a gentle current of carbon dioxide. This fraction was oxidized according to the method of Bloor (7) and used for the estimation of the neutral fat by correcting for the total cholesterol present. Total cholesterol was determined colorimetrically by the Liebermann-Burchard method as modified by Bloor (10). Total lipid was estimated as the sum of the phospholipid, neutral fat, and total cholesterol fractions, the values being expressed as mg. per cent.

*Results*

In the four experiments with androstenedione, the dogs were on the beef heart-cracker meal diet and received subcutaneously the requisite dose on three successive mornings. Blood samples were taken from 2 to 4 days before the first injection and then daily with occasional exceptions until 4 to 9 days after the last injections. Dog 2, in two experiments, received daily doses of 20 mg. and 60 mg., Dog 1 received a daily dose of 60 mg., and Dog 6 received 17 mg. the first two mornings and 12.5 mg. on the third.

The injection of androstenedione had no consistent or signifi-

<sup>3</sup> The blood samples were drawn and prepared by H. D. McEwen and C. D. Kochakian.

<sup>4</sup> The analyses were made by P. L. MacLachlan and C. D. Kochakian.

cant effect on the level of the various blood lipids regardless of the nature of the dog—thin castrate, fat castrate, or normal. This was true with respect to both immediate and delayed effects. A period of as much as 9 days after the last injection (Dog 1) showed no noteworthy variations. The constancy of the lipid values was further demonstrated by the fact that similar values were obtained in the two experiments with Dog 2. In the second experiment the animal received 3 times as much hormone as in the first, yet there were no significant differences. The values for Dogs 1 and 2 were very similar to those obtained while on the kitchen scrap diet and are given in Table II. The experiment with Dog 1 is given as representative for the results with androstenedione.

The lipid values for Dog 2 were much higher than those for Dog 1 (*cf.* Table II), while those of Dog 6 were similar to those for Dog 1. These results conform to the nutritive state of the animals (Dogs 1 and 6 thin, Dog 2 fat). The similar values obtained for Dog 1 (castrate) and Dog 6 (normal) substantiate the fact that the nutritive state of the animal rather than castration determines the blood lipid level. Although Dogs 1 and 6 were on the same diet, this cannot account for the similarity in values, as will be seen when we consider the results in Table II.

The values given in Table II were obtained while the dogs were on a kitchen scrap diet which in contrast to the constant diet of Dogs 1, 2, and 6 was relatively high in carbohydrate. The values for Dog 6 are the averages obtained while on the beef heart-cracker meal diet. On comparison of the two diets, the values for Dogs 1 and 2 are quite similar. On the kitchen scrap diet, as on the constant diet, Dog 2 showed similar high values. Dogs 7 to 12, moreover, showed values which are in agreement with their degree of fatness. Of these animals, Dog 12 had very high cholesterol values. The nutritive state of this dog was similar to that of Dog 2.

The duration of castration did not seem to affect the blood lipid values. Dog 1, which was a castrate of 5 years and 1 month, demonstrated blood lipid values analogous to Dogs 7 to 11 which were castrates of only 4 months. Likewise Dog 2, a castrate of 3 years and 1 month, showed lipid values analogous to Dog 12. The nutritive state of these animals, therefore, seems to be the factor determining the blood lipid levels.



On December 9 and 10 Dog 8 received subcutaneously 64 mg. of testosterone oxime and Dog 10 received 25 mg. of testosterone benzoate. In neither instance was there a disturbance in the blood lipid values on the day following the last injection.

#### DISCUSSION

The results obtained in this study indicate that treatment with androgens, as well as castration, does not disturb the lipid metabolism of dogs as indicated by blood plasma values. The differences noted with the various dogs could be related only to the degree of fatness of the animal. The thin dogs showed relatively low values and the fat dogs high values. Even a significant change in the diet failed to cause any noteworthy difference in the blood lipid values.

The failure of the androgens to disturb the lipid metabolism indicates that fatness subsequent to castration is not due to the lack of an adequate supply of these hormones to the animal body but to some other factor or factors.

#### SUMMARY

The injections of androstenedione into a thin castrate, a fat castrate, and a normal dog had no immediate or delayed effect on the blood plasma lipid values—cholesterol, phospholipid, neutral fat, or total lipid. Testosterone oxime and testosterone benzoate, likewise, failed to exert any effects on the level of the plasma lipids of castrate dogs.

No correlation could be shown between the duration of castration or diet on the level of the blood plasma lipids. On the other hand a definite relationship was evident between the nutritive state of the animal and the blood plasma lipid values.

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# OBSERVATIONS ON THE RELATION BETWEEN IONIZED AND TOTAL CALCIUM IN NORMAL AND ABNORMAL SERA AND THEIR ULTRAFILTRATES

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The frog's heart has been found to be a sensitive indicator of the concentration of calcium ions in protein-containing fluids (1). Using the values derived by this method, McLean and Hastings (2) have shown that the total calcium, calcium ions, ( $\text{Ca}^{++}$ ), and total protein of blood serum bear a relationship to one another which apparently follows the law of mass action. Assuming that each molecule of protein acts as a divalent ion towards calcium, and employing the factor 0.243 for base-combining capacity of protein (3), they find that the equation

$$\frac{(\text{Ca}^{++})(\text{Prot}^-)}{(\text{CaProt})} = K$$

describes this relation satisfactorily. The negative logarithm of the dissociation constant ( $\text{pK}$ ) was found to have the approximate value of  $2.22 \pm 0.07$ .

They have further shown that the ( $\text{Ca}^{++}$ ) calculated from the above relation holds for various dilutions of human serum and in various diseases including experimentally induced calcium dyscrasias (4). Since the formulation proposed would greatly simplify the problem of determining the calcium ion concentration of any blood, a simple calculation from the blood calcium and protein levels being all that is necessary, it becomes of great interest to determine whether the relationship holds in all cases. The present cases are submitted because, although few in number, some of them represent relatively unusual disturbances in calcium metabolism.

McLean and Hastings have shown that if all the ultrafiltrable or diffusible calcium is considered as ionized, values for  $pK_{CaProt}$  are ordinarily found to be somewhat lower than those calculated from the results given by the frog heart. The ultrafiltration data from which their calculations were made were not obtained from the serum which was used for the frog heart determinations, nor have calcium ion determinations previously been made on ultrafiltrates. It was therefore thought desirable to carry out both determinations on the same blood. Such procedures would also afford some information pertinent to other assumptions on which the theory of ultrafiltration is based.

### *Methods*

The total calcium on serum, ultrafiltrate, and serum concentrate was determined by the method of Fiske and Logan (5). Total serum protein was determined in most cases by the Kjeldahl method and in a few instances by the Greenberg (6) method.

The methods used for preparing collodion sacs and ultrafiltering the serum were essentially those described by Benjamin and Hess (7). A 30 cc. conical centrifuge tube was used for the ultrafiltrate receiver. The amount ultrafiltered was determined by weighing the ultrafiltration units, with two receivers. With specific gravity determinations on serum, ultrafiltrate, and serum concentrate, these weighings and the calcium analyses afforded a check upon the accuracy of the chemical analyses. To prevent layering of the serum the ultrafiltration units were shaken vigorously at frequent intervals during ultrafiltration.

Calcium ion, ( $Ca^{++}$ ), levels were estimated by the use of the frog heart method described by McLean and Hastings (1). Some difficulty was encountered, especially in the early spring, in obtaining hearts which were of sufficient sensitivity to give accurate readings on sera with high calcium concentrations. Since even the best hearts are likely to be insensitive to changes in ( $Ca^{++}$ ) above 1.5 mM, they are often unsuitable for determinations on markedly hypercalcemic blood. The procedure in this case was to dilute the unknown with an equal volume of calcium-free solution similar to that used in the reference solutions before carrying out the assay. It is obvious, of course, since calcium and protein combine according to mass action, that the calcium ion concen-

tration of the diluted serum will not be one-half that of the pure sample. Calculation of the theoretical value on the basis of the calcium and protein content of the diluted sample may, however, be carried out and comparison then made with the observed value (4). These difficulties do not, however, concern the analysis of the ultrafiltrates in which the  $(Ca^{++})$  concentration may safely be considered to be double that found in the diluted sample.

Hydrogen ion concentration has been found (2) to have little effect on the readings for  $(Ca^{++})$  within the range ordinarily found in human blood. Although we were able to confirm the conclusion that alkalization to the deepest color given by phenol red made no significant differences in the readings, ordinarily all solutions including the unknowns were equilibrated with carbon dioxide and oxygen mixtures so as to obtain a pH of approximately 7.35 as determined by a standard set of buffers containing phenol red. Calculation of serum water was made according to the equation, serum water (in gm. per 100 cc.) =  $99.0 - 0.75P$  ( $P$  = gm. of protein per 100 cc.) (2). The calculation of  $(Ca^{++})$  from the total calcium and total protein present was carried out graphically by means of a nomogram of the equation,

$$\frac{(Ca^{++})(Prot^{-})}{(CaProt)} = 10^{-2.22}$$

### Results

*Comparison of  $(Ca^{++})$  Values Observed and Calculated on Normal and Pathological Sera*—In general the values of  $(Ca^{++})$  observed with the frog heart on normal human sera and on those from patients with various disturbances of calcium metabolism were found to agree closely with those predicted on the basis of the relationship between calcium and protein formulated by McLean and Hastings. In Table I will be found a compilation of all the cases studied. The column headed "observed" contains the determinations by the frog heart method, that entitled "calculated" being the results of the nomographic estimation from the total calcium and protein present in the serum. With the exception of the three cases listed last in Table I, which will be considered separately, agreement between the calculated and observed values of  $(Ca^{++})$  was satisfactory. Calculation of  $pK_{CaProt}$  from the total calcium and observed  $(Ca^{++})$ , with the factors for combining

TABLE I

*Comparison of Calculated and Observed ( $\text{Ca}^{++}$ ) on Normal and Pathological Sera*

Case No.	Total protein	Total calcium		Ca <sup>++</sup> calculated	Ca <sup>++</sup> observed	Ca <sup>++</sup> difference	Remarks
	gm. per 100 cc.	mg. per 100 cc.	mM per kg. H <sub>2</sub> O	mM per kg. H <sub>2</sub> O	mM per kg. H <sub>2</sub> O	mM per kg. H <sub>2</sub> O	
1	7.8	10.3	2.78	1.16	1.02	-0.14	Normal
2	8.3	9.7	2.62	1.02	1.19	+0.17	"
3	7.5	11.6	3.10	1.32	1.28	-0.04	"
4	7.8	10.8	2.90	1.20	1.29	+0.09	"
5	7.9	11.0	2.95	1.22	1.08	-0.14	" fasting
6	8.4	10.4	2.80	1.10	1.08	-0.02	" after breakfast
7	6.1	9.6	2.56	1.23	1.17	-0.06	"
8	8.4	9.5	2.57	1.00	1.08	+0.08	"
9	6.2	9.8	2.60	1.24	1.23	-0.01	"
10	6.4	11.2	3.00	1.42	1.42	0.00	"
11	7.9	10.8	2.90	1.20	1.02	-0.18	"
12	8.4	10.4	2.80	1.08	1.08	0.00	"
13	5.9	17.7	4.70	2.50			Cancer of thyroid with bone metastases
*	2.95		2.30	1.55	1.45	-0.10	Hyperparathyroidism
14	5.8	13.9	3.66	1.88			
*	2.9		1.80	1.20	1.24	+0.04	
15	7.7	14.3	3.84				"
*	3.85		1.84	1.10	1.14	+0.04	
16	7.4	8.3	2.20	0.92	0.85	-0.07	Same after parathyroid removal
17	7.7	7.2	1.93	0.79	0.80	+0.01	Idiopathic tetany (latent)
18	5.5	10.6	2.80	1.45	1.43	-0.02	Hyperthyroidism, basal metabolism +40
19	6.0	10.2	2.70	1.32	1.27	-0.05	Hyperthyroidism, basal metabolism +12
20	7.8	10.8	2.90	1.20	1.07	-0.13	Hyperthyroidism, basal metabolism +40
21	8.2	10.9	2.97	1.20	1.08	-0.12	Myxedema, basal metabolism -10
				Average		-0.03	
Cy	8.2	11.5	3.10	1.22	0.83		
Ax	2.2	9.1	2.38	1.78	0.62		
Sh	2.54	7.7	1.97	1.40	0.62		

\* Frog heart assay carried out on serum diluted with an equal volume of Ca-free Ringer's solution.

power of protein proposed by McLean and Hastings, averaged  $2.23 \pm 0.06$ . This is in close agreement with that found by McLean and Hastings ( $2.22 \pm 0.07$ ).

*Comparison of  $(Ca^{++})$  Values Derived from Ultrafiltration and from Frog Heart Data*—Since it is reasonable to assume that  $(Ca^{++})$  is distributed between the serum and its ultrafiltrate according to the Gibbs-Donnan law, the ratio of  $(Ca^{++})$  in serum to that in the ultrafiltrate depends upon the protein ions present as follows:<sup>1</sup>

$$\frac{(Ca^{++})_s}{(Ca^{++})_{uf}} = \frac{1}{r^2} = 1 + \frac{(\text{Prot})}{(A)_s}$$

where  $(A)_s$  is the total anion concentration of the serum, and  $(Ca^{++})_s$  and  $(Ca^{++})_{uf}$  the calcium ion concentration of serum and ultrafiltrate respectively. Since this equation indicates that, at constant pH and  $(A)_s$ ,  $1/r^2$  is a linear function of the protein concentration and since, according to McLean and Hastings (2),  $1/r^2$  may be assumed to have the value 1.05 when the serum protein concentration is 7 gm. per 100 cc.,  $(Ca^{++})_s$  may be calculated from the determined  $(Ca^{++})_{uf}$  and protein concentration by the equation  $(Ca^{++})_s = (Ca^{++})_{uf} (1 + 0.007 (\text{Prot}))$ . Total serum protein (Prot) is expressed in gm. per 100 cc.

From data existing in the literature, McLean and Hastings (2) have calculated  $pK_{CaProt}$  on the assumption that all the ultrafiltrable calcium is ionized and that  $(Ca^{++})$  is distributed with regard to the membrane according to the relation outlined above. Calculations from 165 experiments comprising both dialysis and ultrafiltration yielded for  $pK$  a rather wide range of values, from 1.32 to 2.55. We have calculated an average value of  $pK$  2.01 from these figures. Similar computations of forty of our cases give a range of values of from  $pK$  1.77 to 2.68, with an average of 2.09 and a median of 2.08. In spite of the wide range of the figures in both these series, some significance may be attached to the mean values in view of the large number of experiments included.

In seventeen of our cases we were also able to carry out frog heart assays of  $(Ca^{++})$  on the ultrafiltrates and, in all but one of these, direct frog heart determinations on the serum are also

<sup>1</sup> This equation is derived by squaring the reciprocal of Equation 34 of the paper by Van Slyke, Wu, and McLean (8).

available. Computation of  $pK$  from values of  $(Ca^{++})_s$  derived from total ultrafiltrable calcium in these cases yielded an average value of  $pK = 2.02 \pm 0.18$ , but if  $(Ca^{++})_s$  was calculated from the  $(Ca^{++})$  as observed on the ultrafiltrate by the frog heart, a higher value of  $pK 2.22 \pm 0.18$  was obtained (Table II). The latter

TABLE II  
Calculation of  $pK_{CaProt}$  from Ultrafiltration Data,  $f Prot = 0.343$

Case No.	Analysis of ultrafiltrate				Ca (frog heart) in ultrafiltrate			
	Total ultrafiltrable Ca (1)	$\frac{1}{r^2}$ (2)	$(Ca^{++})_s^*$ (3)	$pK_{CaProt}$ (4)	$(Ca^{++})_{sf}$ determined (5)	$(Ca^{++})_s$ calculated (6)	$pK_{CaProt}$ (7)	Difference, (1) - (5) (8)
	<i>mM per kg. H<sub>2</sub>O</i>		<i>mM per kg. H<sub>2</sub>O</i>		<i>mM per kg. H<sub>2</sub>O</i>	<i>mM per kg. H<sub>2</sub>O</i>		
1	1.37	105.5	1.45	2.02	0.90	0.95	2.37	+0.47
2	1.35	105.8	1.45	1.92	1.20	1.27	2.05	+0.15
3	1.25	105.2	1.32	2.23	1.20	1.26	2.27	+0.05
4	1.67	105.4	1.76	1.86	1.25	1.32	2.15	+0.42
5	1.55	105.5	1.63	1.96	0.95	1.00	2.37	+0.60
6	1.50	105.9	1.59	1.90	1.05	1.11	2.22	+0.45
10	1.42	104.5	1.48	2.19	1.33	1.39	2.24	+0.09
12	1.14	105.8	1.21	2.15	1.20	1.27	2.11	-0.06
13	2.72	104.2	2.84	2.06	2.20	2.30	2.31	+0.52
14	2.07	104.2	2.16	2.07	2.10	2.20	2.04	-0.03
15	1.45	105.4	1.53	2.29	1.30	1.37	2.38	+0.15
16	1.37	105.4	1.45	1.77	0.65	0.69	2.43	+0.72
17	0.85	105.0	0.89	2.11	0.78	0.82	2.18	+0.07
18	1.40	104.0	1.46	2.21	1.23	1.28	2.33	+0.17
20	1.55	105.4	1.64	1.94	1.12	1.18	2.24	+0.43
21	1.40	106.0	1.48	2.05	0.90	0.95	2.35	+0.50
22	1.37	104.8	1.44	2.08	1.25	1.31	2.17	+0.12
Average.....				2.02 $\pm 0.18$			2.22 $\pm 0.18$	
Median.....				2.06			2.24	

\* All ultrafiltrate considered ionized.

value agrees satisfactorily with the results obtained by frog heart assay directly on the serum (Table I). The figures for both total ultrafiltrable calcium and the  $(Ca^{++})$  of the ultrafiltrate are also given in Table II, Columns 1 and 5, and reveal that whenever significant differences appear between the two (Column 8) the

total ultrafiltrable calcium is higher, as would, of course, be expected.

On the face of it this seems to indicate that there is, at least in some sera, a significant amount of calcium bound to something other than protein. It is unlikely, however, that this can account for all the variations recorded. In our series there is some reason to suppose that the difficulty of obtaining accurate analyses on small amounts of ultrafiltrate with low concentrations of calcium contributed to some of the discrepant pK values. As a check on our analyses we compared the amounts of calcium found in the serum before ultrafiltration,  $(Ca)_s$ , with the sum of the amounts found inside,  $(Ca)_i$ , and outside,  $(Ca)_o$ , the sac at the end. Such a procedure, of course, involves adding the possibility of several errors, but in general the results confirmed our impression that some of our scattering of pK values could be attributed to analytical difficulties. In general, if the apparent sum of the calcium inside and outside the sac turned out to be greater than the amount in the serum to start with, low values of pK were encountered and *vice versa*; thus the average of the ten lowest pK values in the series was 1.82 and in these the sum of  $Ca_i + Ca_o$  exceeded the  $Ca_s$  by 16 per cent; the ten median values for pK gave an average of 2.08 and here  $Ca_i + Ca_o$  was only 1.6 per cent greater than  $Ca_s$ ; finally the highest ten of the pK values averaged 2.42, and  $Ca_i + Ca_o$  was 11 per cent less than  $Ca_s$ . If most of the error was due to the lack of precise evaluation of  $Ca_o$ , this is the sort of variation which it would introduce in the calculation of the dissociation constant.

Thirty-seven sera including all but three of those on which frog heart assays were also made were analyzed for adsorbable complexes by the method of Benjamin and Hess (7). In view of the theoretically uncertain basis for the adsorption procedures employed (*cf.* Schmidt and Greenberg (9)), a detailed analysis of the results is not included in this paper. In general the relatively wide scattering of the values recorded and the fact that the method ordinarily yielded values for calcium ions (ultrafiltrable non-adsorbable calcium) approximately one-half those obtained by the frog heart were confirmatory of the conclusion (Schmidt and Greenberg (9)) that  $BaSO_4$  is capable of adsorbing relatively large amounts of calcium ion as well as of any hypothetical com-



plex present. Our experiments further indicated that adsorption of calcium stopped short of completion in most instances when the calcium ion level was in the neighborhood of 0.4 to 0.6 mM. Not only is this the case in ultrafiltrates but the calculation of calcium ions from the total non-adsorbable calcium and protein present in serum subjected to  $\text{BaSO}_4$  adsorption indicates that it is true also for serum. The fact that more calcium is adsorbed from serum than from ultrafiltrates is probably due to the greater amount made available by the ionization of  $\text{CaProt}$ , and not because it is present in a specifically adsorbable complex, as supposed by Benjamin and Hess.

#### DISCUSSION

In general, the serum calcium ion concentrations found in our cases by means of the frog heart may be said to show a satisfactory agreement with those previously reported. The method has also been found to be applicable in cases of hypercalcemia from hyperparathyroidism or metastatic carcinoma, as well as in cases of postoperative and idiopathic hypocalcemia.

Some interest attaches, however, to the three cases of Table I in which the calculated and observed ( $\text{Ca}^{++}$ ) did not agree. Two of these cases represent serious degrees of lipid nephrosis. In both, the level of cholesterol in the blood was very high for long periods of time, and the serum was grossly milky in appearance. Not only cholesterol (10) but the fatty acids, and perhaps the phospholipids, cephalin and lecithin, have been shown to have definite effects on the frog heart (11, 12). It seems likely that these or related substances may be responsible for the pressor and toxic effects of some human and animal sera on the frog heart, referred to by McLean and Hastings (1) (and obvious to anyone who has worked extensively with the method). In most cases it is apparently possible to avoid the disturbances introduced by such substances by waiting until the heart has reached a new level after introducing the serum and carrying out one's comparisons with the standard solutions before the effect of the unknown substances has worn off. In the cases of serious lipid disturbances and in case Cy (Table I) such a procedure was, at least in our hands, not satisfactory.

Particular interest attaches to the four cases of thyroid dys-

function reported, since in this condition calcium excretion is greatly altered without an accompanying disturbance in either the protein or total calcium levels in the blood. It seemed possible that it might be accounted for by some disturbance in the ratio of calcium ions to calcium bound to protein or some unknown X substance. In the cases studied no such condition was found, the predictions of the formula derived from normal material adequately describing the calcium ion level found in these cases.

The similarity of the values for calcium ion concentration as found by the frog heart on the various sera and their respective ultrafiltrates is direct evidence that, with the exception of the Donnan effect, calcium ions are distributed in a symmetrical manner on both sides of an ultrafilter. Such a situation has, of course, been predicted heretofore on other grounds, but not experimentally demonstrated. Furthermore, in many cases the values for ionized calcium as found by the frog heart on either serum or its ultrafiltrate do not differ by more than the limit of error from the total ultrafiltrable calcium, so that it is fair to say that in many sera it is unnecessary to postulate the existence of any calcium in a form other than ionic or associated with protein. Nevertheless if the whole series is considered, there is slightly more ultrafiltrable calcium than can be accounted for solely by ( $\text{Ca}^{++}$ ).

In conclusion it is worth while to point out that the great majority of experimental findings may be explained in the single assumption of a calcium-protein equilibrium; it is not necessary to postulate that significant amounts of calcium are combined with substances other than protein. The evidence for a diffusible calcium-phosphorus complex has already been shown to be most uncertain (9). Apparently a non-diffusible phosphate complex may be found both *in vitro* and *in vivo* if grossly abnormal amounts of calcium or phosphorus are suddenly added to blood (Schmidt and Greenberg (9); McLean and Hinrichs (13)), but the evidence available indicates that it disappears from the blood stream within a few minutes after formation. Phosphorus determinations were carried out in roughly one-half of our pathological cases, but none of them was so high as to give a calcium-phosphate product above normal limits. McLean and Hastings state that as much as 2 mm of  $\text{PO}_4$  may be added to their solutions of reference of ionic composition similar to normal blood without diminution of the

calcium ion content except after a period of some hours. A few unpublished experiments by the authors support that conclusion. It seems unlikely, therefore, that phosphate levels influenced the state of the calcium in the blood of any of our cases.

#### SUMMARY

1. Observations on the total calcium, total protein, and total ultrafiltrable calcium and on the ionized calcium of the serum and ultrafiltrates were carried out on a series of normal and pathological sera.

2. Observed values of calcium ion concentration on both serum and ultrafiltrates were found to agree satisfactorily with those calculated on the basis of the calcium-protein equilibrium described by McLean and Hastings.

3. In a certain number of cases more calcium was found in ultrafiltrates than could be accounted for purely as ionized calcium.

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## CAROTENOIDS OF THE CHICKEN RETINA\*

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Many bird and reptile retinas contain brightly pigmented oil globules, situated in the position of light filters between the inner and the photosensitive outer limbs of the cones (Hannover, 1840). Three groups of these, red, golden, and greenish yellow in color, are found in the chicken retina. Schultze (1866) first suggested this arrangement to be concerned in color vision.

Capranica (1877) found the droplets, treated *in situ* with iodine-potassium iodide solution, nitric acid, or sulfuric acid, to yield the violet to green colorations now known to characterize carotenoids. He believed the retinal colors all due to various concentrations of a single pigment, identical with "lutein" from chicken egg yolk, the corpus luteum of the cow, milk, and animal fat.

Kühne and Ayres (1878) separated the chicken retinal pigments into three fractions: purplish red "rhodophane," golden or orange "xanthophane," and greenish yellow "chlorophane." The chlorophane fraction possessed two visible absorption bands; xanthophane and rhodophane each a single band. None of these preparations was pure.

We have separated the retinal pigments into a purplish red acidic, a golden alcohol, and a greenish yellow hydrocarbon fraction. From these we have crystallized three carotenoids, which in suitable solvents reproduce closely the colors of the retinal globules.

### EXPERIMENTAL

Eye tissues were prepared from fresh chicken heads obtained from a slaughter-house. The black choroid tissue was usually

\* This research has been aided in part by a grant from the Milton Fund of Harvard University. A short account of the experiments has appeared in *Nature* (Wald and Zussman, 1937).

included with the retinas, since we found it to contain little or no lipoidal pigment. About 1600 eyes were extracted, in batches of about 100. Each batch was treated in a separate experiment.

Because of the known instability of carotenoid extracts, all operations were conducted as rapidly as possible, and with a minimum of exposure to light. When necessary, preparations were stored on solid carbon dioxide.

*Preliminary Fractionation*—The tissues were shaken repeatedly at room temperature with benzine (petroleum ether, boiling range 30–60°) containing 2 to 5 per cent ethanol. The extract, orange in color, was washed with water. A portion of it was shaken with 90 per cent methanol; all color remains in the benzine (epiphasic).

The benzine was removed under reduced pressure, and the oily residue saponified in 10 cc. of 6 per cent KOH in ethanol, either at room temperature for 3 hours, or at about 40° for 1 hour. The saponification mixture was diluted with an equal volume of water and exhaustively extracted with benzine.

The yellow benzine extract contains hydrocarbon and hydroxy carotenoids. It was washed several times with 5 per cent KOH in 50 per cent ethanol, then several times with water. On being shaken with 90 per cent methanol, a golden alcohol fraction enters the methanol (hypophasic), while greenish yellow hydrocarbon remains in the benzine. Saponification has reversed the behavior of the retinal alcohol in this partition, an indication that it is extracted from the tissues as an ester. The free alcohol is principally epiphasic when shaken with benzine and 80 per cent methanol; it is most probably a dihydroxy carotenoid.<sup>1</sup>

The alkaline saponification residue contains the potassium salt of a red carotenoid. Part of this precipitates as a red powder in the interface during the extraction with benzine. It may be redissolved in the aqueous layer by adding more ethanol or KOH; or it may be driven into benzine by acidifying slightly. It is epiphasic in partition between benzine and 90 per cent methanol,

<sup>1</sup> The general basis of these procedures has been summarized by Kuhn and Brockmann (1932) and by Zechmeister (1934). Monohydroxy carotenoids and esters of dihydroxy carotenoids are epiphasic in partition between benzine and 90 per cent methanol. Free dihydroxy carotenoids are hypophasic in this partition, but mainly epiphasic between benzine and 80 per cent methanol. Carotenoids containing more than two hydroxyl groups are mainly hypophasic between benzine and 80 per cent methanol.

but may be withdrawn quantitatively from benzine with 5 per cent KOH in 50 per cent ethanol.

The three fractions which result from this initial procedure are imperfectly separated, and do not exhibit constant spectroscopic properties.

*Further Purification. Hydrocarbon Fraction*—This was adsorbed from benzine as a yellow layer on a column of calcium oxide or activated alumina.<sup>2</sup> It was eluted from the adsorbent with 1 per cent ethanol in benzine. In hexane (boiling range 65–75°) the pigment formed a greenish yellow solution; band maxima of various preparations varied within the limits 416 to 420, 439 to 442, and 468 to 469  $m\mu$ .

*Alcohol Fraction*—This was adsorbed from benzine to form one or more golden layers on a column of calcium carbonate. Each layer was eluted separately with 1 per cent ethanol in benzine. The spectrum of one of these pigments resembles that of lutein (xanthophyll,  $C_{40}H_{54}(OH)_2$ ) in form (Kuhn and Smakula, 1931), but is displaced several  $m\mu$  toward lower wave-lengths. Band maxima of this type of preparation in hexane occurred at about 418, 444 to 446, and 471 to 473  $m\mu$ .

Usually above this pigment on the chromatogram another was adsorbed, which often contained the major portion of the alcohol fraction. In hexane it yielded a curious spectrum, rising by steps into the ultraviolet. Its most prominent band, at about 472  $m\mu$ , agrees in position with the single band of Kühne's xanthophane, with which it probably corresponds. Its spectra in various preparations varied in form, and seem clearly to represent mixtures, though derived from single layers of the chromatogram.

*Acidic Fraction*—This is adsorbed more tenaciously than any of the other retinal pigments on columns of alumina or calcium carbonate. If adsorbed before saponification, it may be eluted with the usual 1 per cent ethanol in benzine mixture; but after saponification alcoholic KOH or glacial acetic acid is required to remove it from alumina. This increase in acidic properties on saponification suggests that it is extracted from the retina as an ester. Adsorption methods proved less satisfactory in the case of this pigment than several alternative treatments.

As already noted, the potassium salt of this pigment precipitates

<sup>2</sup> Activated alumina, grade A, 100 mesh, was obtained from the Aluminum Ore Company, East St. Louis, Illinois.

from dilute aqueous alkali. It can be separated as a scum by centrifuging, the liquor decanted, and the precipitate washed with benzine until the washings are colorless. After slight acidification with acetic acid, the pigment dissolves readily in hexane.

The red pigment precipitates also, partly in microcrystalline condition, from hexane chilled in solid  $\text{CO}_2$ . The precipitate may be washed with benzine, in which it is almost insoluble. It is taken up readily with pyridine.

The absorption spectrum of this pigment consists of a single broad band, maximal at  $472\text{ m}\mu$  in hexane, at  $479\text{ m}\mu$  in chloroform, at  $495\text{ m}\mu$  in pyridine, and at  $506\text{ m}\mu$  in carbon disulfide. Details of these spectra varied slightly in different preparations. Except for the displacement of its spectrum several  $\text{m}\mu$  toward shorter wave-lengths, the properties of this pigment are identical with those of the tetraketo- $\beta$ -carotene, astacene (Kuhn and Lederer, 1933; Karrer, Loewe, and Hübner, 1935).

These procedures yielded therefore three fairly well defined carotenoids, resembling in partition and adsorption properties and spectral form a carotene, a dihydroxy carotenoid or xanthophyll, and astacene. The spectra, however, were not accurately reproducible, and invariably were displaced by appreciable amounts toward shorter wave-lengths than in the familiar carotenoids. In addition a poorly defined alcohol fraction was obtained, probably composed of degraded carotenoid derivatives.

*Crystalline Pigments*—The total pigment from 100 retinas was separated by our preliminary procedure into fractions preferentially soluble in benzine, 90 per cent methanol, and 50 per cent ethanol-KOH.

The hydrocarbon fraction in benzine was transferred to about 0.5 cc. of methanol, and a few droplets of water added. A russet precipitate formed. This was microcrystalline, for (a) the particles appeared luminous when viewed between crossed polarizing screens (polaroid); and (b) they were practically insoluble in methanol, though readily soluble in hexane. Amorphous carotenes are soluble in both. The crystals were washed with 95 per cent methanol. In hexane they form a greenish yellow solution, with bands at 418, 440, and  $469\text{ m}\mu$  (Fig. 1, curve c).<sup>3</sup> Dis-

<sup>3</sup> Spectra shown in this paper were recorded photoelectrically at the Color Measurements Laboratory of the Massachusetts Institute of Technology, with the instrument of Professor A. C. Hardy (1935).

solved in castor oil, used to simulate the retinal oil, this pigment yielded spectrum *c* of Fig. 2, with maxima at 427, 450, and 480  $m\mu$ . The form of these spectra is typical of carotenes, but their maxima lie at much lower wave-lengths than those of the common carotenes. The properties of this pigment are almost identical with those of the hydrocarbon sarcinene, derived from the bacterium *Sarcina lutea* (bands at 415, 440, and 469  $m\mu$  in petroleum ether) (Chargaff and Dieryck, 1932).

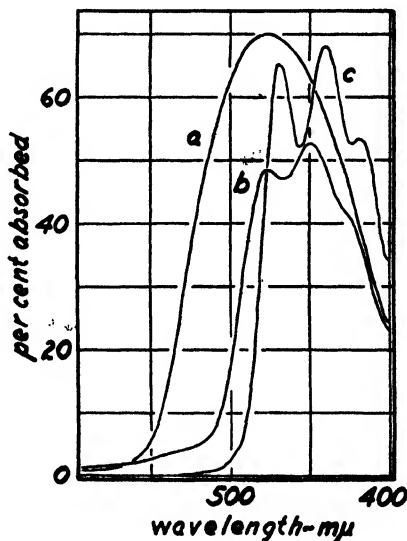


FIG. 1. Spectra of crystalline retinal astacene (*a*), xanthophyll (*b*), and hydrocarbon (*c*) in hexane.

The alcohol fraction in 90 per cent methanol was diluted with water to a methanol content of about 80 per cent, the pigment extracted with benzine, and transferred to about 0.5 cc. of methanol. On addition of a little water a heavy orange-red microcrystalline precipitate appeared. The crystals were washed thoroughly with hexane, in which they are quite insoluble. They dissolve readily in methanol. The spectrum of this pigment possesses maxima at about 420, 450, and 477.5  $m\mu$  in hexane (Fig. 1, curve *b*), at about 450, 482, and 510  $m\mu$  in carbon disulfide, and at about 430, 463, and 490  $m\mu$  in castor oil (Fig. 2, curve *b*). This is intermediate between the spectrum of lutein (447.5 and 477.5  $m\mu$  in



benzine; 445, 475, and 508  $m\mu$  in carbon disulfide) and that of its isomer, zeaxanthin (424, 452, and 485  $m\mu$  in benzine; 452, 484, and 520  $m\mu$  in carbon disulfide). It probably represents a mixture of both pigments, like that found in the chicken egg (Kuhn and Smakula, 1931).

The potassium salt of the red pigment was precipitated by dilution of its alkaline solution with water. The precipitate was washed with benzine until the washings were colorless. It was dissolved in about 0.5 cc. of methanol, acidified with a little glacial

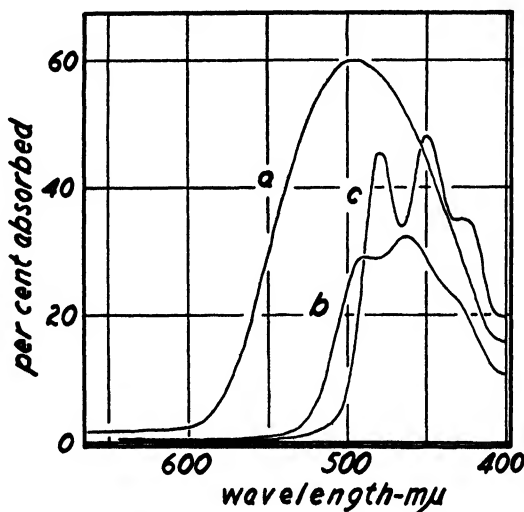


FIG. 2. Spectra of crystalline retinal astacene (a), xanthophyll (b), and hydrocarbon (c) in castor oil.

acetic acid, and a few droplets of water added. Crystals of astacene appeared, thin flat purple blades, arranged about a center (Fig. 3, A). The crystals were insoluble in hexane and methanol. They dissolved easily in pyridine, from which minute amounts were recrystallized on microscope slides as fine needles (Fig. 3, B). In pyridine this pigment possesses the single maximum at about 500  $m\mu$ , characteristic of astacene (Kuhn and Lederer, 1933). In hexane the band maximum lies at about 477  $m\mu$  (Fig. 1, curve a); in castor oil at about 497  $m\mu$  (Fig. 2, curve a).

It is possible that only these three crystalline pigments occur

in the retina, and that in spite of our precautions the other preparations were partly degraded in the course of treatment. Carotenoids are known to be particularly labile in dilute solutions such as those with which we worked. The inconstancy of spectra of the non-crystalline preparations shows none of them to have been quite pure. Yet degraded pigments may have been present in the retina itself, and for this reason all the results may be significant.

*Vitamin A*—The carotenoid derivative, vitamin A, occurs in the retinas of a number of marine fishes, frogs, and mammals, as an integral component of the rhodopsin system (Wald, 1934-35,

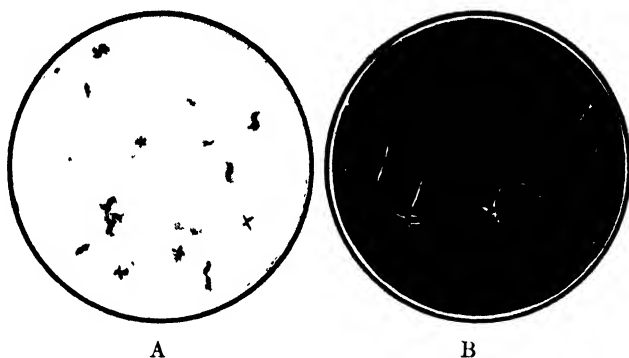


FIG. 3. Crystals of retinal astacene, (A) from methanol-acetic acid; and (B) from pyridine, the latter photographed between crossed polarizing screens (polaroid).

1935-36, 1936-37). Rhodopsin has been found so far only in the retinal rods. Relatively few rods occur in the chicken retina, and attempts to identify rhodopsin in it heretofore have failed (Kühne, 1878).

We have attempted to recognize vitamin A spectrographically in antimony trichloride tests with whole extracts of chicken retinas, and with extracts partly freed by saponification and adsorption of retinal acids and xanthophylls. These tests yielded blue colorations characteristic of carotenoids, but not the band at 615 to 620  $m\mu$  which identifies vitamin A. Spectra of the antimony trichloride reaction with a total extract of forty retinas are shown in Fig. 4. Curve 1 was recorded within the first minute

after mixing the reagents, Curves 2 and 3 several minutes later. There appears to be an initial band at about  $625\text{ m}\mu$ , superimposed on general absorption in the red. This cannot be identified at present with the reaction of any known carotenoid.

*Ontogeny of Retinal Pigments*—Schultze (1867) observed in an unidentified variety of chicken that red retinal droplets appear in the embryo on the 18th and yellow droplets on the 19th day of incubation. In an "Italian race" and in the Wyandotte, Hahn (1916) found colored globules to appear on the 16th and to be

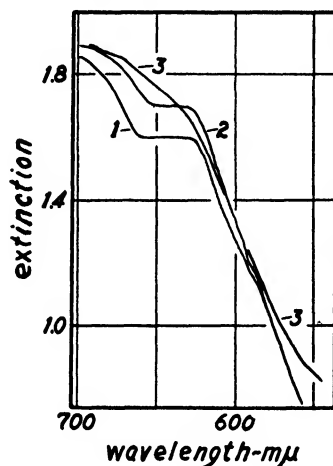


FIG. 4. Spectra of the antimony trichloride reaction with a total extract of chicken retinas. Curve 1 was recorded within the 1st minute after mixing the reagents, Curves 2 and 3 several minutes later. The ordinates are recorded as extinction,  $\log I_0/I$ , in which  $I_0$  is the incident and  $I$  the transmitted intensity.

fully differentiated on the 17th day. Walls and Judd (1933) found colorless droplets to appear in the white Leghorn on the 14th day, red on the 15th, and yellow-orange on the 17th day of incubation.

We have followed the development of colored retinal globules in Rhode Island Red embryos, incubated at  $37^\circ$ . Two eggs were examined each day following the 10th. Colorless droplets appeared on the 15th day, pink early on the 19th, yellow droplets later.

Clearly the retinal pigments appear in chick embryos well

before hatching, and must be derived either by synthesis or from the carotenoids of the yolk.

*Carotenoids in Other Tissues*—Of the three crystallized retinal carotenoids, only the xanthophyll has so far been recognized in other tissues.

Chicken egg yolk contains a number of carotenoids, varying with the diet. Lutein and zeaxanthin (Kuhn, Winterstein, and Lederer, 1931), cryptoxanthin,  $\beta$ -carotene, and vitamin A (Gillam and Heilbron, 1935) have been identified. Our examination of yolk pigments revealed neither the retinal hydrocarbon nor any trace of astacene.<sup>4</sup>

Chromatographic analysis of an extract of chicken livers on alumina yielded a xanthophyll mixture resembling those of yolk and retinas, and a hydrocarbon, the spectrum of which in hexane possessed maxima at 423, 446, and 474 m $\mu$ . This was not an exhaustive analysis; a small amount of the retinal hydrocarbon might also have been present. No trace of astacene was found.

Chicken serum contains a large quantity of xanthophyll which, like carotene in cattle serum (Palmer and Eckles, 1914), apparently is bound to protein. No serum astacene or hydrocarbon was found.

Zechmeister and Tuzson (1934) have found lutein and violaxanthin, principally esterified, in chicken fat deposits. No hydrocarbon was found, nor is any astacene reported.

A cursory search by us for astacene in combs, wattles, and russet-colored feathers also was unsuccessful.

It is concluded that of all the chicken tissues examined, only the retina contains astacene. Absence of the retinal hydrocarbon from other tissues is less certain, since it could more readily have been overlooked.

#### DISCUSSION

*Genesis of Retinal Pigments*—Astacene, and perhaps also the retinal hydrocarbon, appear to be synthesized by the chicken.

<sup>4</sup> We have been informed that chickens allowed to roam sea beaches or given lobster shells as a source of calcium lay eggs with bright reddish yolks, possibly containing astacene. This is a very unusual circumstance, which poultrymen purposely guard against, since it lowers the value of the eggs in the market. The phenomenon itself is not particularly significant, for hens are known to deposit in their egg yolks even so abnormal a dietary component as Sudan III (Palmer and Kempster, 1919).

Neither pigment has yet been identified in the customary chicken diet. The embryonic formation of red retinal droplets in eggs which contain no astacene is strong evidence that this pigment is formed *in situ*. Possibly the retina itself is the site of its synthesis, since astacene is absent from all the other tissues examined.

Astacene is recognized to be a specifically animal carotenoid, typically a marine invertebrate pigment. It has not yet been found in plants. Recently it has been identified in marine fishes, which probably derive it directly from Crustacea in their food (Sørensen, 1935). Its apparent synthesis by a bird is therefore of considerable interest.

*Segregation of Retinal Pigments*—It is clear from the colors of the retinal globules that one type of retinal pigment predominates in each of them. The organism apparently is able to divide these closely related substances fairly efficiently among a mosaic of closely packed cells. The separation of these pigments in the laboratory is greatly aided by the circumstance that one is a hydrocarbon, one an alcohol, and one acidic. A precisely similar situation has been found in the retina of the turtle, *Clemmys insculpta* (Wald, unpublished observations). It is possible that this tripartite differentiation in structure is the chemical basis of pigment segregation in the organism.

*Rôle of Pigments in Vision*—Light which stimulates the chicken cones is first filtered through the three groups of colored retinal globules. A number of theories of the significance of this arrangement have been proposed (see Walls and Judd (1933)); none of them either excludes or improves upon Schultze's suggestion that it may form the basis of color discrimination (1866).

To evaluate the functions of the retinal globules properly it is necessary to know their spectra. Spectra of dissolved carotenoids vary widely with the nature of the solvent, particularly with its refractive index. Most animal and vegetable oils possess refractive indices of 1.46 to 1.48. We have used castor oil of refractive index 1.477 (25°) in an attempt to approximate the retinal condition. The success of this device may be judged partly by comparison with direct spectroscopic observations of the globules by Waelchli (1881), Kühne (1882), and Roaf (1929).

Kühne's measurements are the most complete, and do not disagree with those of the other investigators. Kühne described

maximum absorption in the red droplets at 480 to 490  $m\mu$ , and first (long wave-length) maxima at about 495  $m\mu$  in the orange and 490  $m\mu$  in the greenish yellow droplets. These values are respectively about 10  $m\mu$  lower and 5 and 10  $m\mu$  higher than the corresponding maxima in castor oil of crystalline retinal astacene, xanthophyll, and hydrocarbon. The divergences are not outside the limits of accuracy of Kühne's observations. They may be due in part to some degree of mixture of the pigments in the droplets.<sup>5</sup>

A general requirement for trichromatic vision is that three groups of cones differ in sensitivity to the various portions of the spectrum. This is perhaps accomplished by differences in the absorption spectra of three cone photopigments, or by a single photolabile pigment operating behind three groups of color filters, or by a combination of these devices.

Originally it was thought that color filters for this purpose should transmit widely separated regions of the spectrum, possibly the primaries of the Young-Helmholtz color theory. Perhaps influenced by this idea and the use of insufficiently corrected microscope lenses (see Walls and Judd (1933)), several investigators have described blue and green retinal globules in the chicken (Krause, 1894). Actually it is clear that all the retinal pigments transmit light in roughly the same spectral regions, and their effects upon the spectral sensitivities of the cones must consequently differ little. This fact does not prejudice the possible rôle of the filters in discriminating hues, for Hecht (1931) has shown that the very effective human color vision system probably is based upon very small differences in cone sensitivity.

If the chicken does depend upon the globule pigments for color differentiation, it should be unable to discriminate hues between about 600 and 700  $m\mu$ , for none of the pigments in castor oil absorbs appreciably in this region. It is significant that the pigeon, which possesses a probably identical system of cone pigments, cannot discriminate hues between about 615 and 700  $m\mu$  (Hamilton and Coleman, 1933).

<sup>5</sup> The agreement with Waelchli's observations is perhaps more striking. Waelchli found maximum absorption (presumably central maxima) in the red droplets at 500  $m\mu$ , in the orange droplets at 470  $m\mu$ , and in the yellow droplets at 450 to 460  $m\mu$ . The central maxima of crystalline retinal astacene, xanthophyll, and hydrocarbon in castor oil occur at 497, 463, and 450  $m\mu$ .

## SUMMARY

Cones of the chicken retina contain oil globules, red, golden, and greenish yellow in color. We have separated retinal extracts into a red acidic, a golden alcohol, and a greenish yellow hydrocarbon fraction. From these, three carotenoids have been crystallized, possessing the properties of astacene, xanthophyll, and an unidentified hydrocarbon.

Neither astacene nor the retinal hydrocarbon could be identified in chicken egg yolk, liver, or serum. Though astacene is absent from the yolk, red retinal droplets appear in chick embryos well before hatching. It is concluded that astacene, and possibly also the hydrocarbon, is synthesized by the chicken.

The possible rôle of these pigments in color vision is discussed.

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# ON OVOVERDIN, THE CAROTENOID-PROTEIN PIGMENT OF THE EGG OF THE LOBSTER\*

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The green pigment of the egg of the lobster (*Homarus americanus*) is a conjugated protein (2, 3). It contains a prosthetic group of carotenoid character which is linked to an albuminoid carrier protein.<sup>1</sup> The pigment was first studied by Newbigin (4) in 1897. The carotenoid component has recently been shown to be an ester of *astacene* (4,5,4',5'-tetraketo- $\beta$ -carotene) with an as yet unknown fatty acid (5-8). The free *astacene* "ovoester" dissolves in organic solvents giving orange to red color. In crystalline form it is insoluble in water. However, under certain conditions colloidal solutions in water may be obtained. The naturally occurring protein complex of the ovoester is grass-green and readily water-soluble. The name *ovoverdin* is hereby proposed for this chromoprotein.

Ovoverdin has been studied with a view to characterizing the protein contained in it and of obtaining information concerning the linkage between the polyene group and the protein component. Inasmuch as ovoverdin is readily obtained in large quantities and is comparatively stable, it was hoped that it might provide a convenient chemical model for visual purple which, according to the interesting experiments of Wald (9), is likewise a carotenoid-protein pigment.

\* A preliminary note dealing with some results of this work has been published (1). This investigation was aided in part by a grant from the Elizabeth Thompson Science Fund to which grateful acknowledgment is made.

<sup>1</sup> For literature on carotenoid-protein complexes reference is made to the monographs by Palmer (2) and by Zechmeister (3).



## EXPERIMENTAL

*Preparation and Purification of Ovoverdin*—Undeveloped lobster eggs are used as starting material. They are ground in a mortar with the aid of purified quartz sand. Distilled water is added and the dark green solution is decanted from the insoluble residue and the sand. An equal volume of saturated ammonium sulfate solution is added. Small amounts of globulins are thereby precipitated. Sometimes oil globules containing free carotene separate from the mixture. Filtration yields dark green, clear solutions. They remain practically unchanged for several weeks if kept in the dark and at low temperatures. Later on, decomposition and formation of a yellowish pigment occur. Such solutions contain no other substance absorbing light specifically in the visible region besides ovoverdin. They were therefore used for the majority of the experiments here reported. A further purification of the pigment may be effected by saturating the solutions with ammonium sulfate which will precipitate ovoverdin quantitatively. After filtration the pigment is dissolved in distilled water. This operation has been carried out six times on one pigment sample. The electrolytes may be removed by dialysis through cellophane at low temperature.

*Quantitative Preparation*—The eggs contain 34 per cent solids. Extraction with distilled water dissolves 38 per cent of the solids. Treatment of the aqueous extract with an equal volume of saturated ammonium sulfate solution removes 6.4 per cent of the proteins, presumably globulins. The dialyzed solution contains after filtration 71 per cent of the proteins present before dialysis. Since full saturation with ammonium sulfate is required for the precipitation of these proteins they may be classified as albumins or albuminoid proteins. This fraction contains practically all of the ovoverdin present in the eggs.

*Analysis of Preparations*—The pigment concentration of the ovoverdin stock solutions was determined by means of the Lange photoelectric colorimeter.<sup>2</sup> To 1.0 cc. of the solution to be analyzed, 3 cc. of pyridine were added with stirring; pyridine

<sup>2</sup> This instrument, as well as the other photoelectric apparatus and galvanometers used in this work, was kindly placed at the disposal of the authors by Pfaltz and Bauer, Inc., New York.

breaks up the carotenoid-protein complex, precipitates the protein, and dissolves the ovoester. The resulting solution, after filtration and dilution with pyridine to 10 cc., was compared with a standard solution of 2.6 mg. of crystalline ovoester, prepared according to Kuhn and Lederer (5), in 10 cc. of pyridine.

The results of the protein and ovoester determinations are summarized in Table I.

*Effects of Various Reagents on Ovoverdin. Organic Solvents*—Ethyl alcohol causes a pink precipitate. Pyridine precipitates the protein and dissolves the ovoester with red color. Acetone produces an orange precipitate and dissolves the ovoester with orange color. Chloroform, upon shaking, causes a color change to red and coagulates the protein. The same is true with benzene. Petroleum ether does not affect the green pigment. Dilute formaldehyde causes a very slow color change to red (in

TABLE I  
*Analysis of Ovoverdin Preparations*

Preparation No.	Total protein content per cc.	Astacene-ovoester per cc.
	mg.	micrograms
III	44	52
IV	42	57
V	38	63

the course of several hours), while concentrated formaldehyde produces the same change within 5 minutes.

*Acids and Alkali*—Mineral acids and acetic acid produce an immediate color change from green to red. *Upon neutralization with alkali the change is found to be irreversible.* Boric acid was found ineffective. Sodium or potassium hydroxide solutions have the same effect as mineral acids. Dilute ammonium hydroxide solution has no appreciable effect. Concentrated ammonium hydroxide produces a color change to brown-olive. Some of these color changes have previously been noted (4, 8).

*Reducing and Oxidizing Agents*—When ovoverdin solutions, buffered by phosphate or bicarbonate, are treated with solid sodium hydrosulfite, the green color persists, though it appears to become somewhat less intense. Hydrogen, activated by colloidal palladium, has no effect. If an excess of 30 per cent

hydrogen peroxide is added, there is no immediate change, but in the course of 1 hour the color turns from green to orange and a protein precipitate forms.

*Miscellaneous Reagents*—Ovoverdin is stable in phosphate and acetate buffer solutions ranging from pH 4 to 8. Sulfosalicylic acid and trichloroacetic acid produce orange-red precipitates. Ovoverdin is precipitated by saturating its solutions with ammonium sulfate, sodium sulfate, magnesium sulfate, and ammonium chloride. The precipitate redissolves in distilled water.

*Adsorptive Properties of Ovoverdin*—Freshly prepared aluminum hydroxide gel, but not Lloyd's reagent, fullers' earth, or kaolin, adsorb ovoverdin from solutions containing ammonium sulfate (pH 5.2). Water, 1 per cent secondary sodium phosphate solution, 20 per cent glycerol solution, with and without addition of dilute ammonia, and egg albumin solution are ineffective as elutriants.

*Effect of Light on Ovoverdin*—A solution of ovoverdin, containing 10 per cent ammonium sulfate and 10 mg. of protein per cc., was irradiated in the concentrated light of a 500 watt projection lantern which had passed a cell filled with water. To one sample a few drops of a solution of lactoflavin were added. After 3.5 hours the dark control was unchanged, the irradiated flavin-free solution was brown, and the irradiated flavin-containing solution was light yellow-brown. It follows that ovoverdin is slowly bleached under these conditions, and that the rate of bleaching is accelerated by lactoflavin.<sup>3</sup>

5 mm. thick gelatin films containing ovoverdin were prepared. If such films are exposed to daylight for 1 to 2 days, the color changes from grass-green to a pale yellow, while the dark control retains the original color.

*Effect of Temperature on Ovoverdin*—If ovoverdin solutions, containing ammonium sulfate, are slowly heated in a water bath, the color changes listed in Table II are observed.

*If a sample is quickly heated to 65–70° and if, after the color has changed from green to orange-red, the solution is quickly cooled to room temperature, the green color reappears.* During the cooling intermediate tints are observed. This color cycle may be re-

<sup>3</sup> About the possible rôle of a flavin in the bleaching of visual purple in the retina see Chase (10).

peated several times with the same sample. If, on the other hand, the heating is carried out slowly so that the pigment is exposed to high temperatures for about 10 to 20 minutes, no reversal of the color change takes place upon cooling. Once coagulation of protein has occurred (at temperatures from 85–100°), the color change is invariably found to be irreversible. In the absence of neutral salts the color change is likewise irreversible; dialyzed ovooverdin solutions show no reversal upon quick heating and cooling. An ammonium sulfate concentration of 0.1 to 0.6 M is most favorable for reversal. The ammonium salt may be re-

TABLE II  
*Effect of Temperature on Ovooverdin*

Temperature	Color	Transparency
°C.		
25	Grass-green	Clear
48	Green with olive tint	"
52	Olive	"
56	" with brown tint	"
60	Olive-brown	"
62	Brown-yellow	"
64	Brown-orange	"
68	Orange	"
72	Deep orange	Faint turbidity
75	Orange with red tint	" "
81	Orange-red	" "
83	"	Increased turbidity
85	"	Strong turbidity
90–100	"	Orange-red coagulum forms

placed by sodium sulfate or potassium chloride. Oxygen is not required for the phenomenon; the cycle has been observed in an atmosphere of pure nitrogen.

The reversible color change upon heating and cooling of ovooverdin in presence of ammonium sulfate produces no significant change in hydrogen ion concentration (before the experiment, pH 5.20, after the experiment, pH 5.28, as measured with the glass electrode).

If ovooverdin solutions are maintained at various temperatures, ranging from 40–70°, for periods of 10 to 180 minutes, gradual

color changes take place after the solutions have acquired the temperature of the bath. In order to follow these changes objectively, the solutions were placed in an absorption cell of 20 mm. depth. The cell was immersed in a trough equipped with glass windows. The water in the trough was stirred and kept at the desired temperature by a microburner. The light of a 250 watt projection lamp was condensed to a beam of about 3 cm. diameter. The beam passed a diaphragm and then the solution in the absorption cell. Behind the trough there was placed a differential selenium rectifier cell (Lange) with a red and a blue glass filter (Corning G 34-R, 3 mm., and Corning G 584-J, 5 mm., respectively) in front of the two halves of the cell. The two negative terminals of the cell were connected with each other, and the two positive terminals were equipped with leads to a multiflex galvanometer (Lange). The lower sensitivity range of the galvanometer, *i.e.*  $4 \times 10^{-7}$  ampere per scale division, was used. The ovoverdin stock solution Preparation IV (for analysis, see Table I) was diluted 5 times with water, bringing the salt concentration to 0.6 M. With this solution in the beam of light, the deflection of the galvanometer which registers only the difference in photoelectric current between the two cell halves was brought to zero by adjusting the distance of the photocell from the trough and by focusing the lens of the projection lamp. When the bath had been adjusted to the desired temperature, the absorption cell was immersed in it and the changes in photoelectric current with time were noted along with the subjective color changes. Fig. 1 contains the results obtained in these experiments. It is seen from the curves in Fig. 1 that at first there is a more or less rapid change in transmission, depending on the temperature selected. Later on, the curves flatten out. The pigment solution acquired the bath temperature after about 5 minutes. The final transmission levels are reached the more quickly the higher the temperature. On the right-hand side of Fig. 1 the color of the pigment solution is given. No abrupt changes indicative of reactions occurring in distinct steps are observed. The transition of the reversible to the irreversible stage occurs very gradually.

In order to obtain an estimate of the extent of reversibility of the color change under favorable conditions, the 20 mm. absorp-

tion cell was replaced by a test-tube of 1 cm. diameter. 5 cc. of ovoerdin Preparation V, diluted 1:1 with water, were placed in the tube and the deflection of the galvanometer was adjusted to zero. For these experiments the sensitivity of the galvanometer was increased 10 times compared with that used in the ex-

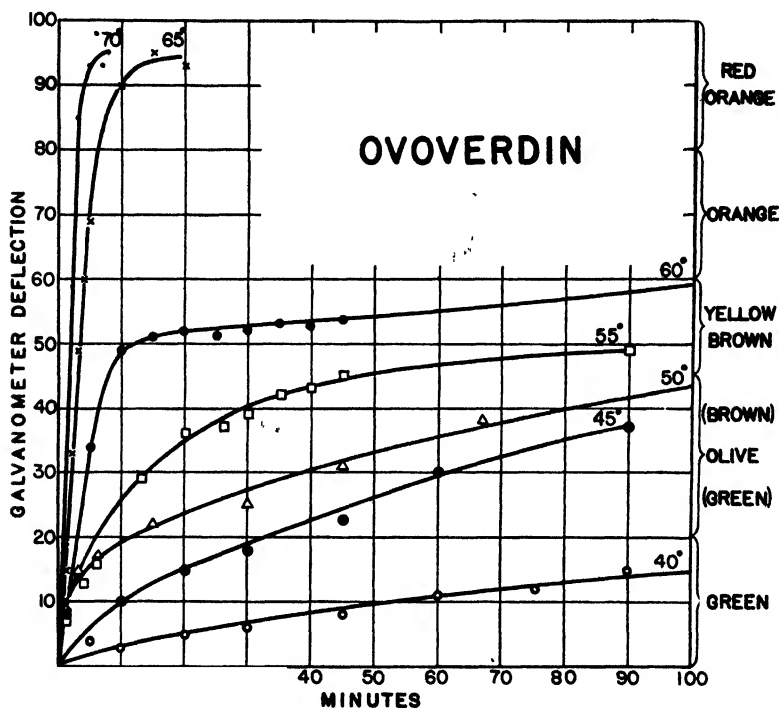


FIG. 1. Time-temperature-color curves of ovoerdin. Abscissa, length of time for which the ovoerdin solution was kept at the temperatures indicated on the individual curves. Ordinates, left, galvanometer deflections, given as scale divisions, as a measure of the extent to which the differential photoelectric cell was thrown out of balance owing to the color changes; right, colors observed visually.

periments represented in Fig. 1; one scale division corresponded now to  $4 \times 10^{-8}$  ampere. The tube was removed from the holder and placed in a water bath of 70°. The temperature of the solution reached 68° within 80 seconds. The tube was put back in the optical tract and permitted to cool down to room

temperature. After 10 minutes the solution had resumed its green color, the temperature was 29°, and the deflection of the galvanometer was fourteen scale divisions instead of zero, as before the experiment. In another experiment the tube, instead of being permitted to cool slowly, was rapidly cooled to room temperature in a cold water bath after being heated to 68°. While there was no deflection before the beginning of the experiment, the final deflection after the first cycle was fourteen scale divisions, after the second cycle twenty-three and a half scale divisions, and after a third cycle twenty-seven scale divisions. These measurements indicate that the color change upon rapid heating and cooling is largely but not completely reversible.

*The reversible and the irreversible color change of ovoverdin with change in temperature is not confined to solutions of the pigment but may also be observed with intact lobster eggs.*

**Absorption Spectrum of Ovoverdin**—The absorption spectrum of ovoverdin was determined in the Color Measurements Laboratory of the Massachusetts Institute of Technology by means of the recording spectrophotometer of Hardy. An ovoverdin solution containing 19 mg. of astacene ovoester per liter, measured at 31° in a 10 mm. layer, gave Curve A of Fig. 2. The cell was then heated at 70° for 3 minutes, and the resulting orange-red form of the pigment was measured (Fig. 2, Curve B). The determination required about 4 minutes, during which time the solution had cooled to 50°; a control experiment showed that the temperature of the solution was approximately 61° while the region of the absorption band at 480 m $\mu$  was being measured; the temperature had fallen to about 55° when the region around 620 m $\mu$  was measured. A third measurement was made when the solution had again reached room temperature (Fig. 2, Curve C). No further change was observed after a subsequent interval of 30 minutes.

It will be seen from Fig. 2 that the green form of the pigment has two absorption maxima in the visible region, at 470 m $\mu$  and at 645 m $\mu$ . In the red form the absorption in the blue region is increased by about 13 per cent, with the maximum shifted to 485 m $\mu$ , the absorption in the region around 640 m $\mu$  having fallen to about one-half of the value observed for the green form. The curve obtained after cooling lies intermediate between those of

the green and red forms. The relatively small extent of reversal in this experiment may be ascribed to too long a period of heating and to the fact that the salt concentration (1 M ammonium sulfate) was not optimal.

Satisfactory agreement with the above curves was obtained by means of the visual spectrophotometer of Bausch and Lomb.

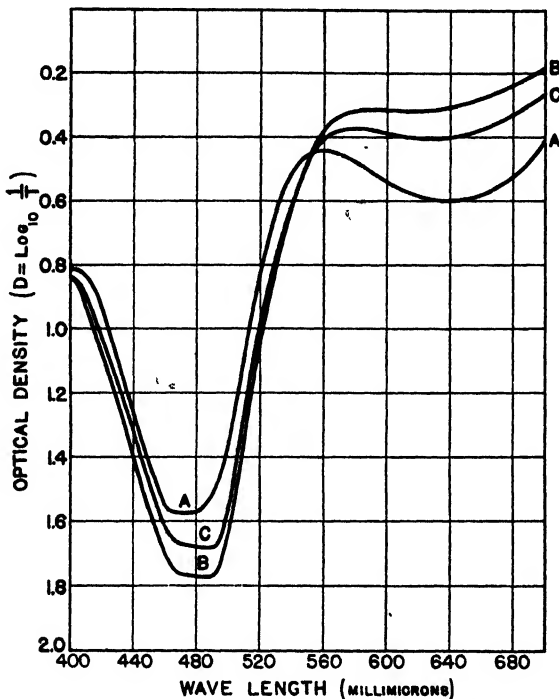


FIG. 2. Light absorption curves of ovoerдин traced by the recording photoelectric spectrophotometer of Hardy. Curve A, ovoerдин at room temperature; Curve B, ovoerдин after heating to 70°. During the tracing of this curve the solution cooled to about 50°. Curve C, the same solution after about 20 minutes, when it had again reached room temperature.

As shown by Wyckoff (11), ovoerдин, like other proteins, absorbs ultraviolet light in the range 230 to 270 mμ.

*Extinction Coefficients of Ovoerдин*—The properties of ovoerдин solutions may be defined in terms of their content in ovoester representing the prosthetic group of the pigment (*cf.* Table I).



Assuming that one of the formulas proposed by Karrer and Hübner (8), namely  $C_{40}H_{47}O_4 \cdot C_8H_{15}O$ , is correct, a molecular weight of 718 would follow for the ovoester. It is possible, however, that this value may be as large as 800.

The curves shown in Fig. 2 were obtained with an ovoverdin solution containing 19 mg. of astacene ovoester per liter. The layer of thickness of the solution was 1 cm. With the equation proposed by Drabkin and Austin (12),

$$\epsilon (c = 1 \text{ mm per liter}) = \frac{\epsilon \text{ observed}}{d(\text{depth})_{\text{cm.}} \times c (\text{actual concentration in mm per liter})}$$

where  $\epsilon$  observed is equal to the density values taken from Fig. 2, and assuming a molecular weight of 718 for the ester, the following extinction values are obtained for the wave-lengths corresponding to the absorption maxima of the two forms of ovoverdin.

$$\begin{array}{ll} \text{Green form, } 470 \text{ m}\mu = 60, & 645 \text{ m}\mu = 22.7 \\ \text{Red form, } 485 \text{ " } = 68, & 620 \text{ " } = 12.3 \end{array}$$

*Molecular Weight*—The fact that ovoverdin contains a protein component is already indicative of a considerable molecular weight of the pigment. This is further suggested by the inability of ovoverdin to pass cellophane or collodion membranes.

The preliminary determination of the sedimentation constant of ovoverdin, carried out by Wyckoff (11) with the aid of the analytical air-driven ultracentrifuge, yields values in the neighborhood of 11, indicating a molecular weight of the order of 300,000. At the time of these measurements it was found that the preparations contained no other pigment besides ovoverdin and that the amount of ultraviolet-absorbing material remaining in solution upon sedimentation of ovoverdin was probably less than 10 per cent of the total protein content. The photographs made with blue light yield the same sedimentation constant as those made with ultraviolet light. The sedimenting substance responsible for light absorption in these regions is therefore the same.

*Isoelectric Point*—The isoelectric point of ovoverdin may be determined by cataphoresis. A small cell, suitable for qualitative experiments, is used and the directions by Michaelis and

Rona (13) are followed. Dialyzed ovooverdin solutions, containing approximately 30 micrograms of combined astacene ovoester per cc. were used. The sense of migration of the boundary of the pigment layer and the approximate mobility are determined visually. The direction of migration of ovooverdin is reversed between pH 6.6 and 6.8. The isoelectric point must, therefore, lie within this range.

#### DISCUSSION

*Constitution of Ovooverdin*—The protein to which the astacene ovoester is linked in ovooverdin imparts an albuminoid character to the pigment. It is soluble in distilled water and it is precipitated by full saturation with ammonium sulfate. Ovooverdin forms large molecules. The sedimentation constant, as determined by Wyckoff (11), indicates a molecular weight of about 300,000, which is nearly 5 times larger than that of hemoglobin (68,000). The isoelectric point of ovooverdin (near pH 6.7) is very close to that of oxyhemoglobin (pH 6.8). The number of prosthetic groups present in 1 ovooverdin molecule may roughly be estimated as follows: The ovooverdin preparations obtained in this work contained an average of 41 mg. of total protein and 57 micrograms of astacene ovoester per cc. After dialysis, the total protein content is 29 mg. per cc. The photographs obtained in the ultracentrifuge indicate that not less than 90 per cent of the ultraviolet light-absorbing material is ovooverdin; *i.e.*, not less than 26 mg. per cc. With a molecular weight of 300,000 for ovooverdin, 651 gm. of ovoester would be contained in 1 mole of the pigment. This figure is so close to the probable simple molecular weight of the ovoester (700 to 800) that the conclusion appears justified that 1 molecule of astacene ovoester is present per molecule of ovooverdin. 1 molecule of hemoglobin contains four hematin groups (14), while 1 molecule of the yellow enzyme contains one lactoflavin phosphoric ester grouping (15).

With respect to the stability of the linkage between the prosthetic group and the protein component, ovooverdin is more similar to the yellow enzyme than to hemoglobin. Organic solvents or heat coagulation will not disrupt the combination of heme with globin, whereas in the carotenoid-protein and in the yellow enzyme (15) the linkage is broken by such treatment.

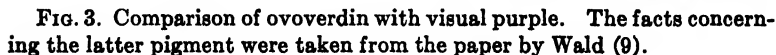
*Thermal Dissociation of Ovoverdin*—Three stages may be distinguished in the course of heat treatment of ovoverdin. (1) A largely reversible change occurring upon rapid heating to not over 70° and subsequent rapid cooling. A color cycle (green, at room temperature, → orange-red, at 65–70°, → green, after cooling to room temperature) is observed during these operations. *The presence of neutral salts is required for reversibility.* (2) An irreversible color change from green to orange-red taking place if the heating is continued for some time at 65–70° or if it is carried out in salt-free solution. (3) Between 90–100° coagulation takes place. The entire carotenoid component is present in the precipitate. These observations are interpreted as follows: Step (1) represents a reversible thermal dissociation of ovoverdin into the astacene ovoester and the free protein carrier. The orange-red color observed at high temperatures is that of the carotenoid group which presumably exists in colloidal solution. Upon cooling, the two components recombine to form the original pigment complex (reversal of color to green). In step (2) the protein carrier is changed (perhaps denatured) and no recombination with the carotenoid takes place upon cooling. Step (3) represents the precipitation of the denatured protein in the presence of neutral salts (heat coagulation).

The phenomenon of reversible heat dissociation is not limited to carotenoid-protein complexes. With oxidized cytochrome C, which represents a combination of a modified hematin with a low molecular protein, it may be observed under much more drastic conditions (Keilin and Hartree (16)).

If the protein component of the yellow ferment is brought to 38°, it loses the ability to recombine with lactoflavin phosphoric acid. This inactivation is slowly reversed at room temperature (Theorell (17)).

*Comparison of Ovoverdin with Visual Purple*—Visual purple, according to Wald (9), is a combination of a carotenoid, retinene, with a protein carrier. When visual purple is exposed to light, there is a rapid change to yellow (visual yellow) and a subsequent slow change to colorless (visual white) which will take place even in the dark. The course of events, especially in solution, is probably more complex than here indicated (18). The primary change from visual purple to visual yellow, according to Wald,

## LOBSTER EGG



It has been inferred by Wald (9) and postulated by Mirsky (20) that the liberation of retinene from visual purple by light is due to a *denaturation* of the protein component. However, the critical increment of heat denaturation of visual purple is about

75,000 calories per mole (9), whereas the energy content of the light at the effective wave-length of 5300 Å. is only 53,000 calories per quantum.

The present observations on ovoverdin suggest that the rupture of a carotenoid-protein complex does not necessarily involve protein denaturation. It may be, therefore, that the primary "bleaching" of visual purple is not a light denaturation of the protein carrier but a photodissociation of the type of the reversible thermal dissociation of ovoverdin.<sup>4</sup> Such a concept would help to explain why the visual cycle proceeds in visible light, at an energy level below that of protein denaturation.

The behavior of ovoverdin and of visual purple under various conditions is summarized in Fig. 3.

#### SUMMARY

1. The green pigment of the egg of the lobster has been studied as a representative of the class of carotenoid-protein pigments. The prosthetic carotenoid group is an ester of astacene (Kuhn, Karrer). For the combination of the astacene ovoester with the protein the name *ovoverdin* is proposed.

2. The protein component of ovoverdin has an albuminoid character. The molecular weight of ovoverdin has been found in the ultracentrifuge by Wyckoff to be approximately 300,000; the isoelectric point, as determined by cataphoresis, is near pH 6.7. The absorption spectrum of the native pigment in the visible region shows maxima at 640 and 470 mμ. There is also light absorption in the ultraviolet.

3. The effect of reagents, of light, and of heat on ovoverdin has been studied. Organic solvents (except petroleum ether), acids, and alkali coagulate the protein and liberate the carotenoid component. Visible light bleaches ovoverdin slowly to straw-yellow. Ovoverdin exhibits phenomena of reversible and irre-

<sup>4</sup> In this connection the observations of Yamamoto (21) on the eggs of *Ceratocephale osawai*, a polychete worm, are of interest. The eggs, which are lemon-yellow in color, become green upon illumination by a tungsten filament lamp. When kept in the dark, the original lemon-yellow color returns in the course of several hours. Yamamoto has carefully studied the light and dark reactions involved in this remarkable phenomenon. The authors are indebted to Dr. George Wald for directing their attention to this publication.

versible thermal dissociation which may be studied with the aid of the accompanying color changes.

4. The relationship between ovoverdin and visual purple is discussed and the possible significance of the present observations for the theory of the bleaching of visual purple is pointed out.

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## A SIMPLE SYNTHESIS OF *dl*-CITRULLINE

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In 1930 Wada (1) established the structure of citrulline isolated from watermelon juice as  $\alpha$ -amino- $\delta$ -carbamido-*n*-valeric acid. A few years later the same author (2) reported its occurrence in protein, a finding not yet substantiated by other workers, although Klein and Tauböck (3) had earlier indicated their belief that citrulline was a constituent of proteins of the Cucurbitaceæ. In addition to its possible presence in proteins the importance of citrulline in the scheme for urea formation proposed by Krebs and Henseleit (4) has made desirable a convenient preparation of this interesting compound which is so closely related both to arginine and to urea. It is the purpose of this paper to describe a simple synthesis of *dl*-citrulline from *dl*-ornithine by utilizing the capacity of copper to form complexes with the two substances. A satisfactory preparation of the intermediate *dl*-ornithine mono-sulfate from  $\alpha$ -carbamylarginine is also described.

Wada (1) synthesized citrulline by hydrolyzing ornithuric acid to  $\alpha$ -monobenzoylornithine which was then condensed with urethane to give  $\alpha$ -monobenzoylcitrulline. Following its isolation the latter compound was hydrolyzed by acid to benzoic acid and citrulline, the citrulline being thereafter obtained as the insoluble copper compound. Assuming a 100 per cent yield in the benzoylation of ornithine to give ornithuric acid, the over-all yield of citrulline from ornithine was less than 8 per cent. The  $\alpha$ -monobenzoylcitrulline has been prepared also by heating urethane,  $\alpha$ -monobenzoylornithine, and an acidic chloride, such as zinc chloride or ferric chloride, to 150° in the absence of water (5). This alternative method, of course, has not eliminated the

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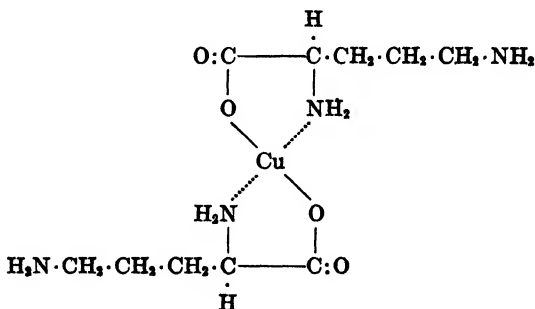


undesirable necessity for the intermediate preparation of  $\alpha$ -monobenzoylornithine. The attempted preparations of citrulline derivatives directly from those of arginine (6, 7) have given results of theoretical interest but of no practical importance in so far as the preparation of citrulline itself is concerned. Furthermore, neither natural sources (1, 2) nor the putrefactive decomposition of arginine (8) gives high yields of the desired compound.

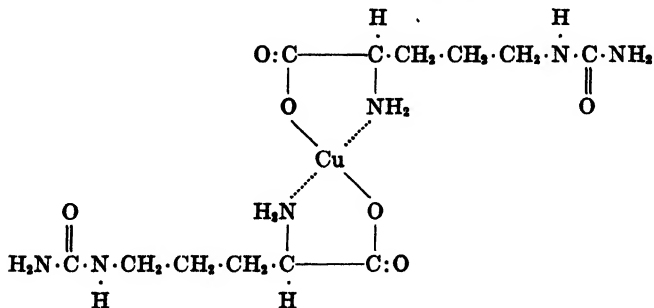
It appears that one of the chief difficulties with the older methods of citrulline synthesis lies in the low yields of  $\alpha$ -monobenzoylornithine obtained by the prolonged action of a boiling barium hydroxide solution upon ornithuric acid. The preparation of  $\alpha$ -monobenzoylornithine by this method was originally proposed by Sørensen, Höyrup, and Andersen (9) and later slightly modified by Wada (1) and by Boon and Robson (10). By isolation and repeated treatment of the unchanged ornithuric acid the first authors obtained a total yield of 60 per cent of the theory; with one 20 hour period of boiling Wada obtained 41 per cent. Using increased concentrations of barium hydroxide, Boon and Robson (10) claimed higher yields (76 per cent) after a 15 hour hydrolysis. The writer's attempt to duplicate the latter result with smaller quantities gave 41 per cent of the monobenzoylornithine, together with considerable quantities of the benzoylaminopiperidone found under somewhat similar circumstances by Thomas, Kapfhammer, and Flaschenträger (11). It seems, therefore, that none of these methods for the preparation of  $\alpha$ -monobenzoylornithine is entirely satisfactory. The synthesis of citrulline described below has obviated the difficulty by allowing a direct preparation from ornithine itself without the necessity for the intermediate preparation of ornithuric acid,  $\alpha$ -monobenzoylornithine, and  $\alpha$ -monobenzoylcitrulline.

The present synthesis of *dl*-citrulline is effected by converting *dl*-ornithine monosulfate into ornithine copper sulfate and by subsequently heating the aqueous solution of the latter with an excess of urea in a sealed tube at 100°. The precipitated citrulline copper is removed from the cold tube and decomposed in the usual manner by hydrogen sulfide to give 65 to 71 per cent of the theoretical amount of the free amino acid. Besides eliminating considerable labor the new method has thus given 9 times more *dl*-citrulline, based on the ornithine taken, than did Wada's method (1).

The formation of copper complexes by the  $\alpha$ - and  $\beta$ -amino acids and the failure of the  $\gamma$ -,  $\delta$ -, and  $\epsilon$ -amino acids to give such compounds (12), together with the generally accepted views on the structure of these and analogous complexes (13), lead one to expect that in ornithine copper the  $\alpha$ -amino group will be protected, while the  $\delta$ -amino group will be free to react with appropriate reagents. It is assumed that in neutral or alkaline solution the ornithine copper possesses the chelate structure here shown.



Provided it is sufficiently stable, this complex will fulfil the necessary conditions in masking the  $\alpha$ -amino group and in not interfering with reactions at the  $\delta$ -amino group. The fact that citrulline copper is of very low solubility makes it seem probable that if urea and ornithine copper are condensed in hot concentrated aqueous solution the citrulline copper complex



will be precipitated as soon as appreciable amounts of it are formed, and that thereafter it will be impossible for urea to react with the protected  $\alpha$ -amino group of the chelate compound. The experiments described below support these views.

The successful masking of the  $\alpha$ -amino group in the present case raises the question whether the intermediate formation of chelate compounds may not be applied in a wide variety of synthetic procedures. Specifically, the method used for the synthesis of citrulline would appear to be a general one for the preparation of  $\alpha$ -amino- $\omega$ -carbamido derivatives of normal acids from the corresponding diamino acids, provided only that, as indicated by considerations outlined in the preceding paragraph, the acid contains at least 4 carbon atoms. Preliminary small scale experiments on the condensation of urea with *dl*-lysine copper chloride have yielded a substance possessing the properties expected for  $\epsilon$ -carbamyllysine but whose identity is not yet firmly established.

#### EXPERIMENTAL

*$\alpha$ -Carbamylarginine*—The  $\alpha$ -carbamylarginine used in the preparation of ornithine was prepared from hydrolyzed gelatin by the convenient method of Boon and Robson (10, 14). A solution of arginine hydrochloride was prepared through the flavianate from the hydrochloric acid hydrolysate of 400 gm. of the cheapest available air-dried gelatin. The neutralized colorless solution, which contained considerable sodium chloride, was concentrated *in vacuo* to 160 cc. and treated 1 hour on the steam bath with 28 gm. of potassium cyanate and 1 gm. of urea. The urea was added because previous experiments had shown its beneficial effect in decreasing the tendency, noted first by Boon and Robson (14), for  $\alpha$ -carbamylarginine to form supersaturated solutions. Placed in the cold room overnight, the mixture gave 29.3 gm. of colorless  $\alpha$ -carbamylarginine which on one recrystallization melted at 174°.

*dl*-Ornithine Monosulfate—Boon and Robson (10) noted the increased yields of ornithine (isolated as ornithuric acid) obtainable with increased concentrations of the alkali used to hydrolyze arginine or  $\alpha$ -carbamylarginine. These authors also noted the detrimental effect of the large amounts of barium carbonate formed when barium hydroxide was used as the hydrolytic agent and therefore suggested the use of sodium hydroxide. However, when the latter base is used, the salicylaldehyde procedure (15) must be resorted to if salts of ornithine itself are to be

isolated. Since it seemed desirable to use as few steps as possible in the preparation of the ornithine salt, the method here described was followed.

25 gm. of  $\alpha$ -carbamylarginine were hydrolyzed by boiling with a solution of 200 gm. of barium hydroxide octahydrate in 250 cc. of water. The mixture was boiled in a 500 cc. Kjeldahl flask containing a few jagged bits of porcelain and equipped with a reflux condenser protected by a soda lime tube. The barium carbonate which precipitated was filtered off from the hot mixture at the end of 1, 2, 4, and 7.5 hours. Each precipitate was washed twice on the filter with boiling water and then suspended in hot water and boiled out twice. The combined wash fluids thus obtained each time were rapidly concentrated to a small volume over a flame, filtered, and added to the original hydrolysis mixture. This procedure was adopted in order to minimize losses due to adsorption on the precipitate. The hydrolysis was interrupted at the end of 15.5 hours, although ammonia was still being evolved and barium carbonate was being precipitated at a slow rate. The barium remaining in the solution was precipitated by passing carbon dioxide through the warm, diluted hydrolysis mixture. After refrigeration and removal of the granular barium carbonate the filtrate was concentrated *in vacuo* at bath temperatures below 55°. The resulting solution was made up to exactly 100 cc. and a 10 cc. aliquot reserved for the preparation of characteristic ornithine derivatives. The larger portion (90 cc.) was converted into ornithine monosulfate (16) by exactly neutralizing the basic solution to nitrazine with sulfuric acid. A scanty precipitate of barium sulfate was removed by filtration through an asbestos mat and the ornithine salt precipitated from the clear and colorless filtrate by the addition, with stirring, of several volumes of 95 per cent ethanol. Crystallization started in the first gummy precipitate within a few minutes. Following refrigeration and filtration the pure white, crystalline *dl*-ornithine sulfate amounted to 14.2 gm. (76 per cent of the theoretical) after drying over sulfuric acid. Other runs have given yields up to 82 per cent. The salt melted at 230–231° with decomposition and with some previous browning; Vickery and Cook (16) reported *dl*-ornithine monosulfate as melting at 234° with effervescence, darkening slightly above 225°. The *dl*-ornithine sulfate described above con-

tained a slight amount of ash, but one recrystallization (yield 12.9 gm.) from water with the addition of ethanol reduced the ash to less than 0.1 per cent. Polariscopic observation of an 8.37 per cent solution in a 20 cm. tube failed to show any significant optical rotation, thus indicating a completely racemized product.

A portion of the 10 cc. aliquot was converted into the picrate which melted at 206–207° with gas evolution; Vickery and Cook (16) give 208° as the decomposition point for *dl*-ornithine dipicrate. A second portion of the small aliquot was benzoylated to give *dl*-ornithuric acid melting at 183–185° after a single recrystallization from aqueous ethanol. This melting point agreed with that obtained for ornithuric acid previously prepared according to the method of Boon and Robson (10).

*dl*-Citrulline Copper—*dl*-Ornithine monosulfate (2.5 gm.) was dissolved in water (15 cc.) and boiled gently for 30 minutes with an excess (1 gm.) of black copper oxide. In other runs the boiling was restricted to 10 or 15 minutes. Urea (3.2 gm.) was added to the deep blue solution of ornithine copper obtained after filtering off the excess copper oxide and the whole concentrated on the steam bath to about 10 cc. During the concentration a negligible amount of bluish copper carbonate was precipitated, as small amounts of ammonia were lost from the hot solution. The hot concentrated mixture was transferred to a glass tube, sealed in, wrapped in a cloth, and heated in a boiling water bath for 3 hours. At the end of this time the contents of the tube had set to a mushy mass of the blue citrulline copper complex. Heating merely in a beaker placed on the steam bath was ineffective in bringing about the desired synthesis. Thus an earlier experiment in which the urea was so heated in the ornithine copper solution for more than 8 hours gave no citrulline copper. The sealed tube was opened after refrigeration overnight. There was generally a little pressure in the tube, but in some runs, particularly in those in which considerable free space remained in the tube, slight negative pressure was observed. The citrulline copper was filtered off with suction and thoroughly washed with water and finally with alcohol. Dried in air for 24 hours, the product weighed 2.03 gm. An additional 0.07 gm. was obtained by reheating the filtrate for 8 hours longer. The final yield was thus 74 per cent. Citrulline copper from different

runs varied somewhat in color; when the reactants were present in more dilute solution, the color tended to be deeper blue.

*dl-Citrulline*—2.03 gm. (97 per cent of the total) of citrulline copper were suspended in 30 cc. of water and treated with hydrogen sulfide. The resultant copper sulfide was very fine but could usually be removed by filtration through a thin washed layer of infusorial earth. When this procedure gave a turbid filtrate, a drop of dilute hydrochloric acid was added and the whole refiltered through a layer of the earth to give a clear solution. The citrulline solution was concentrated to a small volume *in vacuo* and several volumes of ethanol added. After the crystallization was well started, a little ether was added and the whole placed in the cold room overnight. Filtration and subsequent washing with 95 per cent ethanol gave 1.51 gm. of pure white citrulline which melted at 218–220° with browning and gas evolution. The total yield of *dl*-citrulline was thus 65 per cent of the theoretical, based on the ornithine monosulfate weighed. Other runs gave yields up to 71 per cent.

On one recrystallization from water with the addition of ethanol the citrulline melted at 220–221° with decomposition; mixed with citrulline (m.p. 220° with decomposition) previously prepared through  $\alpha$ -benzoylornithine and  $\alpha$ -benzoylcitrulline, the melting point was also 220–221°. Like all experimentally determined melting points reported in this paper, these values are corrected for thermometer stem exposures. It may be noted that the melting point found agrees much better with the 220–222° reported for citrulline by Ackermann (8) than with the 205–206° first reported by Wada (1) or with 226° as later reported by the same author (2). The citrulline crystals resembled those figured by Wada (1) and dissolved in water to give a neutral solution with a faintly sweet taste. The material was insoluble in ethanol and in ether. Dried over phosphorus pentoxide at room temperature the substance gave the following analyses.

$C_5H_{11}N_2O_3$ .	Calculated.	C 41.11, H 7.48, N 23.99
	Found.	" 41.04, " 7.38, " 23.46 (micro-Dumas)
		" 23.56 (macro-Kjeldahl)

#### SUMMARY

A convenient method of preparing ornithine monosulfate from  $\alpha$ -carbamylarginine is described. Ornithine copper sulfate has

been condensed with urea in hot concentrated aqueous solution to give citrulline copper in good yields. The further use of chelate formation in organic syntheses is suggested.

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## THE EXTRACELLULAR AND INTRACELLULAR WATER IN BONE AND CARTILAGE

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(Received for publication, September 28, 1937)

In this study on human fetal bone and cartilage the calculations determining extracellular and intracellular water are based on the assumption that some electrolytes are practically limited to the cells and others to the extracellular body fluids. Recently Harrison, Darrow, and Yannet (1) showed that more sodium is present in the skeleton of the rabbit, dog, and monkey than can be accounted for by the extracellular water of the bone and cartilage. They showed also that the total body sodium of the human fetus was greatly in excess of the sodium calculated for the extracellular water, particularly during the later months of fetal life. If this sodium excess is characteristic of body growth, calculations from the composition of fetal bone and cartilage should show a similar trend.

### *Methods*

The tissues for the analyses were obtained at autopsy at the Lying-In Hospital, within 8 to 16 hours after death of the fetus. Postmortem changes were minimized as far as possible by refrigeration until the time of autopsy. The femur and tibia were freed from all connective tissue, the epiphyseal cartilage was snapped off, and the weight of the moist bone obtained as rapidly as possible. The cartilage was dissected out to avoid calcified areas, freed from all connective tissue, and weighed. Both bone and cartilage were dried to constant weight at 105°. The bone was pulverized in a mortar, and bone ashings were made in platinum dishes in a muffle furnace at 450°. Because of the flint-like nature of the dried cartilage, the cartilage was not pulverized. Approximately 1 gm. quantities were ashed in platinum dishes at 450°.



Analyses were made of powdered, dried, whole bone including trabeculae and marrow. Cartilage was analyzed separately.

TABLE I

*Calculations of Extracellular and Intracellular Water of Fetal Bone*

Fetus No.	Birth weight	Bone	Analytical data*			Calculated data		
			Water	Total sodium	Total chloride	Extra-cellular water	Intra-cellular water	Sodium of extra-cellular water
	gm.		per cent	m.-eq. per kg. fresh tissue	m.-eq. per kg. fresh tissue	per cent	per cent	m.-eq. per kg. fresh tissue
11	650	Femur	52.4	130.5	52.4	43.6	8.8	64.6
16	660	"	46.4	142.8	45.9	38.2	8.2	56.6
13	760	"	45.6	138.3	45.2	37.7	7.9	55.8
13	760	Tibia	43.9	155.0	50.3	41.9	2.0	62.0
18	800	Femur	48.8	141.4	50.5	42.1	6.7	62.3
18	800	Tibia	47.1	151.8	50.8	42.3	4.8	62.6
10	920	Femur	46.1	119.3	41.1	34.3	11.8	50.8
10	920	Tibia	43.9	125.1	47.5	39.6	4.3	58.6
8	1230	Humerus	39.7	148.4	46.3	38.6	1.1	57.2
8	1230	Femur	42.7	149.5	48.6	40.5	1.2	60.0
5	1300	"	46.9	163.8	48.9	40.7	6.2	60.2
5	1300	Tibia	47.7	161.8	46.3	38.6	9.1	57.1
21	1310	Femur	41.9	146.8	39.8	33.2	8.7	49.1
21	1310	Tibia	41.7	151.3	38.6	32.2	9.5	47.7
22	1680	Femur	41.9	151.6	42.0	35.0	6.9	51.8
22	1680	Tibia	41.6	154.8	46.3	38.6	3.0	57.2
12	1910	Femur	42.6	151.3	41.6	34.7	7.9	51.4
12	1910	Tibia	42.4	158.5	45.6	38.0	4.4	56.2
15	2050	Femur	46.2	139.3	35.8	29.8	16.4	44.1
15	2050	Tibia	46.6	142.6	41.9	34.9	11.7	51.7
3	2340	"	41.9	163.1	44.8	37.3	4.6	55.2
25	2790	Femur	43.3	155.4	38.7	32.2	11.1	47.7
9	3030	"	44.4	145.4	39.6	33.0	11.4	48.9
9	3030	Tibia	44.1	146.8	42.1	35.1	9.0	52.0
6	3360	"	39.6	154.0	37.2	31.0	8.6	45.9
14	3800	Femur	45.3	148.8	36.3	30.2	15.1	44.7
14	3800	Tibia	42.4	157.2	42.2	35.2	7.2	52.1
26	4030	Femur	44.5	145.1	33.7	28.1	16.4	41.6

\* From tables by the authors (9).

Fat was not removed, because it was thought the action of 95 per cent alcohol might remove some of the sodium from the

cartilage. However, seven extractions of dried bone for 48 hour periods in Soxhlet extractors gave an average total fat value of 4.6 per cent; of the two cartilage samples analyzed, fat values of 9 and 12 per cent were obtained. Chlorides were determined directly on the dried bone and cartilage by the method described by Logan (2), sodium in the ash solution by the method of Barber and Kolthoff (3) as modified by Butler and Tuthill (4), potassium by the method of Fiske and Litarczek (5), and magnesium by the method of Fiske and Logan (6).

The method of calculating the values presented in Tables I to IV is that used by Harrison, Darrow, and Yannet (1) who assume that practically all body chloride is a constituent of extra-

TABLE II  
*Summary of Calculations of Extracellular and Intracellular Water of Fetal Bone*

Birth weight of group	No. in group	Analytical data			Calculated data		
		Total water of bone	Total sodium	Total chloride	Extra- cellular water	Intra- cellular water	Sodium of extra- cellular water
gm.		per cent	m.-eq. per kg. fresh tissue	m.-eq. per kg. fresh tissue	per cent	per cent	m.-eq. per kg. fresh tissue
650- 920	8	46.8	138.3	48.0	40.0	6.8	59.2
1230-2340	13	43.4	152.8	43.4	36.1	7.3	53.4
2790-4030	7	43.4	150.5	38.6	32.1	11.3	47.5

cellular water and potassium and magnesium are in the intracellular water. These assumptions are subject to known errors in that 5 milli-equivalents of potassium and 1 to 2 milli-equivalents of magnesium are present per liter of extracellular body fluid and a small amount of chloride is present in the erythrocytes of the vascular system in bone. Average values of 148 and 120 milli-equivalents per liter (1, 7, 8) were assumed for the sodium and chlorine of extracellular fluid; values of 35 and 140 milli-equivalents per liter (1) for the magnesium and potassium of intracellular fluid. Because of difficulties encountered in obtaining duplicate chloride values on samples of dried cartilage, calculations of extracellular and intracellular water of cartilage are based on the potassium and magnesium contents.

TABLE III  
*Calculations of Extracellular and Intracellular Water of Epiphyseal  
 and of Costal Cartilage\**

Fetus No.	Weight	Analytical data			Calculated data		
		Cartilage water	Total sodium	Total potassium	Extra-cellular water	Intra-cellular water	Sodium of extra-cellular water

Epiphyseal cartilage							
	gm.	per cent	m.-eq. per kg. fresh tissue	m.-eq. per kg. fresh tissue	per cent	per cent	m.-eq. per kg. fresh tissue
11	650	85.5	161.7	56.0	45.5	40.0	67.4
16	660	86.5	138.5	60.0	43.6	42.9	64.5
13	760	85.3	161.0	56.2	45.2	40.1	66.9
18	800	86.7	165.5	49.9	51.1	35.6	75.6
4	810	85.6	167.3	51.4	48.9	36.7	72.4
10	920	85.3	194.0	47.7	51.2	34.1	75.8
8	1230	83.7	179.2	58.1	42.2	41.5	62.4
5	1300	84.7	135.0	63.7	39.2	45.5	58.0
21	1310	85.2	162.7	56.5	44.8	40.4	61.9
22	1680	85.6	191.5	41.2	56.2	29.4	83.2
12	1910	85.5	197.8	42.8	54.9	30.6	81.2
15	2050	84.3	177.4	55.0	45.0	39.3	66.6
3	2340	83.6	172.2	49.9	48.0	35.6	71.0
9	3030	83.9	173.9	44.9	51.8	32.1	76.6
6	3360	82.1	146.9	62.3	37.6	44.5	55.6
14	3800	82.7	177.8	61.8	38.6	44.1	57.1
1	4150	82.5	215.3	38.5	55.0	27.5	81.4
Average.....		84.6	171.6	52.7	47.0	37.6	69.6

Costal cartilage							
4	810	83.6	150.5	70.2	33.5	50.1	49.6
10	920	83.2	186.1	74.8	29.8	53.4	44.1
5	1300	82.7	114.8	65.2	36.1	46.6	53.4
3	2340	80.0	161.3	62.6	35.3	44.7	52.2
9	3030	81.4	136.3	61.2	37.7	43.7	55.8
6	3360	79.3	132.7	70.8	28.7	50.6	42.5
1	4150	81.7	135.8	71.6	30.6	51.1	45.3
Average.....		81.7	145.3	68.1	33.1	48.6	49.0

\* Based on the assumption that all potassium is intracellular and equivalent to 140 milli-equivalents per liter of intracellular water.

*Results and Comments*

In Table I are shown the data on extracellular and intracellular water for fetal bones calculated on the assumption that practically all the chloride is in the extracellular fluid. It is apparent from the analytical data that there is little change in the sodium content per unit weight of bone during fetal growth. There is, however, a marked decrease in the chloride content. The volume of the extracellular fluid calculated from the chloride values decreases accordingly. The sodium in excess of that required for tissue fluids increases in proportion to the loss of extracellular fluid. If the basis of these calculations is valid, it appears that as growth progresses less and less sodium is held in the extra-

TABLE IV  
*Summary of Calculations of Extracellular and Intracellular Water of Fetal Cartilage*

Cartilage	No. of specimens	Fresh tissue	Cartilage water	Extracellular water	Intracellular water
		<i>m.-eq. per kg.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Epiphyseal	15	Cl = 50.1	84.0	41.8	42.2
	17	Mg = 12.7	84.6	48.3	36.3
	17	K = 53.2	84.6	46.6	38.0
Costal	3	Cl = 34.7	80.2	28.9	51.3
	7	Mg = 16.8	81.7	33.7	48.0
	7	K = 68.1	81.7	33.1	48.6

cellular phase and more is gathered into the complex inorganic salts of the matrix of the bone. Table I also shows that the intracellular water of the tibia is generally much lower than that of the femur.

Table II is a summary of Table I. A comparison of the first and last groups shows an increase in sodium of 9 per cent, a fall in chloride of 20 per cent, and in extracellular water and extracellular sodium a fall of 20 per cent. There is a 66 per cent increase in the calculated volume of intracellular water from the 6th to the 10th fetal months.

In Table III are presented data from calculations of extracellular and intracellular water of epiphyseal and costal cartilage based on the assumption that practically all potassium is found

in the intracellular water. Cartilage differs from bone in that no progressive change in fluid phases is to be noted during fetal growth.

Table IV is a summary of data on fetal cartilage. With one exception the values for extracellular and intracellular water are in good agreement whether chlorine, magnesium, or potassium is the basis for the calculations.

#### SUMMARY

1. Calculations for extracellular and intracellular water of fetal bone and cartilage of eighteen fetuses weighing 650 to 4030 gm. are presented.

2. The distribution of extracellular and cellular water in fetal cartilage is not materially changed whether chloride, potassium, or magnesium is the basis for the calculations.

3. In bone the sodium content per unit weight of fresh tissue increases very little during fetal growth. The changes noted in the extracellular and cellular water are almost entirely determined by the progressive fall in chloride.

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## AMINO ACID CATABOLISM

### V. THE INFLUENCE OF STRUCTURAL CONFIGURATION ON THE DEAMINATION OF $\alpha$ -AMINO ACIDS IN THE NORMAL DOG\*

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We have shown (Leighty and Corley, 1937) that in the normal dog, after subcutaneous injection, amino acids with straight chains yield their nitrogen as urea readily, while those with a methyl group on the same carbon atom as the amino group lose their nitrogen with difficulty. Observations with *dl*-valine could best be interpreted on the basis that spatial configuration was of importance in determining the influence on deamination of a methyl group on the carbon atom adjacent to the one with the amino group. More definite evidence to confirm this view now has been obtained from studies with the separate isomers of valine and of isoleucine. To extend our knowledge of deamination, the fate of each of a number of additional compounds has been followed.

After the urinary nitrogen of a dog maintained on a complete diet (Cowgill, 1923) became reasonably constant, in aqueous solution, the substance to be studied was injected subcutaneously, or in a few instances given orally. Evidence as to its fate is furnished by the change or lack of change in the total nitrogen (Kjeldahl), urea and ammonia nitrogen (Van Slyke and Cullen), and amino acid nitrogen (by formol titration, Van Slyke and Kirk (1933)). The return of the urinary values afterwards to

\* A report of this work was presented before the Thirty-first meeting of the American Society of Biological Chemists at Memphis, April, 1937.

Based on a thesis submitted by Fred H. Snyder to the Faculty of Purdue University in partial fulfilment of the requirements for the Degree of Doctor of Philosophy, June, 1937.

TABLE I

*Disposal in Urine of Nitrogen of Subcutaneously Injected Amino Acids*

Experiment No.	Day	Total N	Urea N	Ammonia N	Amino acid N	Compound administered
		gm.	gm.	gm.	gm.	
1	14	4.40	3.72	0.23		
	15	4.32	3.40	0.45	0.15	
	16	4.86	3.59	0.45	0.54	0.57 gm. N as d(-)-valine
	17	4.36	3.51	0.41	0.13	
	18	4.40	3.36	0.48		
2	17	3.17	2.65	0.18		
	18	3.23	2.79	0.25	0.08	
	19	3.52	3.01	0.30	0.07	0.60 gm. N as l(+)-valine
	20	3.13	2.71	0.24	0.10	
	21	3.24	2.79	0.23		
3	22	4.66	3.69	0.37		
	23	4.73	3.76	0.42	0.13	
	24	5.35	4.33	0.44	0.13	0.53 gm. N as l(+)-isoleucine
	25	4.66	3.81	0.37	0.15	
	26	4.50	3.67	0.34		
4	11	4.21	3.68	0.23		
	12	4.19	3.64	0.24	0.10	
	13	4.95	3.65	0.26	0.59	0.53 gm. N as d(-)-isoleucine
	14	4.11	3.53	0.28	0.11	
	15	4.07	3.33	0.29		
5	18	3.38	2.81	0.28		
	19	3.36	2.61	0.36	0.10	
	20	3.93	3.27	0.34	0.09	0.43 gm. N as l(-)-leucine
	21	3.47	2.89	0.25	0.08	
	22	3.32	2.60	0.35		
6	24	4.03	3.24	0.52		
	25	4.05	3.24	0.52	0.15	
	26	4.76	4.04	0.44	0.14	0.43 gm. N as d(+)-leucine
	27	4.19	3.28	0.58	0.13	
	28	4.02	3.18	0.52		
7	22	5.52	4.66	0.37		
	23	5.41	4.54	0.44	0.13	
	24	6.36	4.81	0.73	0.54	0.53 gm. N as dl-pseudoleucine
	25	5.47	4.16	0.59	0.14	
	26	5.28	3.48	0.93		
8	14	5.73	4.67	0.32		
	15	5.68	4.69	0.33	0.14	
	16	6.22	4.92	0.42	0.33	0.65 gm. N as dl-phenylglycine
	17	6.30	5.09	0.38	0.12	
	18	5.17	4.16	0.36		

TABLE I—*Concluded*

Experi- ment No.	Day	Total N	Urea N	Ammonia N	Amino acid N	Compound administered
		gm.	gm.	gm.	gm.	
9	37	5.49	4.62	0.51		
	38	5.55	4.60	0.53	0.13	
	39	5.95	4.49	0.70	0.52	0.51 gm. N as <i>dl</i> - allothreonine
	40	5.71	4.75	0.54	0.11	
	41	5.37	4.42	0.41		
10	29	3.93	3.11	0.33		
	30	3.77	3.07	0.35	0.12	
	31	4.61	3.43	0.44	0.47	0.59 gm. N as <i>dl</i> - allothreonine
	32	4.58	3.56	0.46	0.11	
	33	3.75	2.95	0.38		
11	37	4.19	3.46	0.34		
	38	4.13	3.45	0.29	0.13	
	39	4.26	3.62	0.30	0.12	0.42 gm. N as <i>l</i> (+)- alloisoleucine
	40	3.63	3.07	0.33	0.10	
	41	3.83	3.30	0.26		
12	20	3.49	2.78	0.36		
	21	3.46	2.74	0.33	0.09	
	22	3.92	3.23	0.27	0.16	0.50 gm. N as <i>dl</i> - phenylalanine
	23	2.79	2.24	0.27	0.12	
	24	3.43	2.90	0.30		

the levels prevailing before strongly supports the view that the changes are referable to the substance given.

*l*(-)-Leucine and *dl*-pseudoleucine (3,3-dimethyl-2-amino-butanoic acid) have been prepared in this laboratory. Phenylglycine was obtained from the Eastman Kodak Company. We are indebted to Dr. H. E. Carter of the University of Illinois for our supplies of allothreonine and *l*(+)-alloisoleucine. The other compounds have been obtained from Hoffmann-La Roche, Inc. By analysis for amino nitrogen or total nitrogen, all were found to be satisfactory for use without further purification. Except for single experiments with phenylglycine and *l*(+)-alloisoleucine, we have at least a pair of experiments with different dogs for each of the substances. The essential data for a representative experiment with each amino acid are presented in Table I. The column "Day" indicates the time since the dog in each experiment was placed on the complete diet and analyses commenced. The amino acid studied was administered on the 3rd of the 5 days, for which figures are presented in each experiment.



In three experiments we have found that *d*(-)-valine has escaped attack, as shown by lack of increase of urea nitrogen, by increase of amino acid nitrogen, and by isolation of the bulk of the unchanged compound from the urine. The natural *l*(+) form has been catabolized readily. We have noted with interest in four cases of five, that the increases of the total nitrogen and urea nitrogen did not account for the administered nitrogen. As we have found no evidence of renal damage, it may be that this amino acid essential for growth is retained, in part, for some obscure but conceivably important metabolic function in the adult animal. *d*(-)-Isoleucine like *d*(-)-valine escaped attack, after administration on three occasions, while *l*(+)-isoleucine was apparently readily broken down. Both forms of leucine were evidently deaminated readily, though both stimulated excretion of tissue nitrogen.

Knoop and Okada (1923) found the *l* form excreted after the oral administration of *dl*-pseudoleucine. Dakin (1926) found, however, 46 per cent recovery of the unresolved racemic mixture after subcutaneous injection in the rabbit. Our results offer no evidence of significant deamination of pseudoleucine after injection and the portion of the amino acid recovered from the urine was optically inactive. Our results confirm the well known biological resolution and partial deamination of *dl*-phenylglycine. The partition of the extra nitrogen in the urine indicated that only part of the mixture was deaminated, while the optical activity of the material isolated from the urine showed that it was the levorotatory form that escaped catabolism. After oral or parenteral administration, *dl*-phenylalanine has yielded most of its nitrogen as urea, while little was excreted unchanged.

Our single observation with *l*(+)-alloisoleucine was less than entirely satisfactory, for although the amino acid nitrogen did not indicate excretion of the unchanged material, the nitrogen of the compound did not all reappear in the urine. However, this result is to be compared with those with *l*(+)-valine. As the results were somewhat different, although indeed of the same import, we record the two experiments which our supply of *dl*-allothreonine made possible. Figures for amino acid nitrogen indicate excretion of the bulk of the compound unchanged. In one study there was excretion of some tissue nitrogen, accounting for the increased urea nitrogen.

## DISCUSSION

We shall assume for convenience of discussion and generalization the not improbable view that the dextrorotatory phenylglycine has the *l*-configuration, like *l*(+)-valine and *l*(+)-isoleucine. The *d* forms of the  $\alpha$ -amino acids studied have not been deaminated significantly unless the  $\beta$ -carbon atom holds 2 hydrogen atoms. The *l* forms, however, have been deaminated readily if the  $\alpha$ - and  $\beta$ -carbon atoms bear 1 hydrogen atom each. This, with the failure of attack of pseudoleucine, might be taken for evidence that deamination proceeds by way of dehydrogenation of these 2 carbon atoms, were it not that *l*(+)-phenylglycine and also glycine readily lose the amino group. The necessity for a hydrogen atom on the carbon atom with the amino group and on the amino group as shown by Friedmann (1908) supports an alternative theory that deamination proceeds by intermediary formation of an imino acid. While the readiness of deamination of *l*(+)-phenylglycine could be explained on this basis, the failure of degradation of pseudoleucine cannot.

The configuration obviously plays an important rôle in deamination, probably by determining affinity to the enzymatic system. Amino acids of the *d* form may then be attacked, if at all, by a group of enzymes different from those for the *l* series. Krebs (1935) found evidence of distinctly different "*l*-amino acid deaminase" and "*d*-amino acid deaminase." However, he found the latter enzyme in tissue slices to attack *d*(-)-valine and *d*(-)-isoleucine, which we find are not attacked in the normal animal under our experimental conditions. Our usual method of administration, subcutaneous injection, might conceivably alter the extent of catabolism, by inducing a rapid rate of excretion. However, *d*(-)-valine and *d*(-)-isoleucine escaped deamination not only after injection but also after oral administration. Our observations do not, therefore, lend support to the view that "*d*-amino acid deaminase" observed in tissue slices operates in the same way or acts on the same compounds in the normal dog.

Results with *dl*-allothreonine are not in accord with those with the other amino acids. We are continuing investigation of the influence of hydroxyl groups on deamination. We have in mind the possibility that deamination may be found to depend on a multiple affinity (*cf.* Bergmann (1935-36)) between enzyme and

substrate and on differing influences of adjacent groups (e.g. benzene ring in phenylglycine).

#### SUMMARY

*dl*-Phenylalanine, *dl*-leucine, *l*(+)-valine, *l*(+)-isoleucine, *l*(+)-alloisoleucine, and *l*(?)(+)-phenylglycine were deaminated readily after subcutaneous injection into the normal dog. *dl*-Pseudo-leucine, *dl*-allothreonine, *d*(-)-isoleucine, *d*(-)-valine, and *d*(?)(-)-phenylglycine were not deaminated significantly after subcutaneous injection into the normal dog.

The following conclusions are drawn for the amino acids studied in the normal dog under the experimental conditions employed.  $\alpha$ -Amino acids of the *l* configuration are deaminated readily if the carbon atom bearing the amino group also has a hydrogen atom and if the  $\beta$ -carbon atom holds a hydrogen atom. Phenylglycine and allothreonine appear to be exceptional.  $\alpha$ -Amino acids of the *d* configuration with a hydrogen atom on the  $\alpha$ -carbon atom are deaminated readily if the  $\beta$ -carbon atom has 2 hydrogen atoms, but not if 1 of these 2 hydrogen atoms is replaced by a methyl group or a hydroxyl group.

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## FURTHER DETERMINATION AND CHARACTERIZATION OF THE COMPONENT ACIDS OF BUTTER FAT

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Interest in milk fats from various sources has centered recently upon the identification of several minor component acids, chiefly unsaturated. The major components are oleic and palmitic acids, representing 50 to 55 per cent of the total mixture of fatty acids (on a molar basis). Butyric, myristic, and stearic acids, contributing 7 to 10 per cent each, account for another 25 to 30 per cent of the total acids. The remaining 20 to 25 per cent is distributed among acids occurring in amounts of 1 to 5 per cent. Of these, the saturated acids, *n*-hexanoic, *n*-octanoic, *n*-decanoic, and lauric, were identified by Crowther and Hynd (7); also, small amounts of saturated acids of higher molecular weight than stearic (*e.g.* arachidic, behenic, lignoceric, and cerotic) were reported by Helz and Bosworth (15). Hilditch and Paul (21) confirmed the presence of myristic and palmitic acids but were unable to isolate lauric acid (*cf.* also Caldwell and Hurlley (6)).

The presence of unsaturated minor component acids of lower molecular weight than oleic was first deduced by Smedley (27). Grün and Wirth (13) confirmed her conclusion with the isolation and identification of  $\Delta^{9,10}$ -decenoic acid. The presence of  $C_{12}$ ,  $C_{14}$ , and  $C_{16}$  unsaturated acids was indicated in later studies by Grün and Winkler (12). Bosworth and Brown (3) presented further evidence for decenoic and tetradecenoic acids but were unable to detect  $C_{12}$  or  $C_{16}$  unsaturated acids. Riemenschneider and Ellis (26) found  $C_{10}$ ,  $C_{14}$ , and  $C_{18}$  monoethenoid acids in goat milk fat; Hilditch and Paul (21) and Longenecker (25) made similar observations when working with butter fat.

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Two other unsaturated acids have been reported as minor component acids of milk fat, (a) a diethenoid  $C_{18}$  acid and (b) arachidonic acid (Brown and Sutton (5) and Bosworth and Sisson (4)). The presence of an octadecadienoic acid has been discussed at length (3, 9, 11, 16). It is clear from the available data that this acid is not identical with the linoleic acid of seed fats. Hilditch (18) has suggested that apparent discrepancies in the results obtained with the octadecadienoic acid of milk fat (cf. also grass glycerides (28) and ox depot fats (20)) may be traced to "different geometrical (*cis-trans*) isomerides of the di-ethenoid acid."

In the present communication we report further evidence for the occurrence in cow's milk fat of decenoic, do-, tetra-, hexa-, and octadecenoic acids, an octadecadienoic acid, arachidonic acid, and the saturated acids from butyric to stearic. Higher saturated acids were not qualitatively examined. We have been especially interested in determining the proportions of the various component acids, using an electrically heated and packed column (25) for the distillations of volatile acids and ester mixtures and comparing these with results previously obtained for cow's milk fats with simpler equipment (Willstätter bulb).

The milk fat studied was a market sample of butter from cows on a normal diet at the National Institute for Research in Dairying, Shinfield, Berks. Its saponification equivalent was 248.0 and iodine value (Wijs) 37.5. Analysis of the component fatty acids was accomplished by methods previously described (19, 22, 23). Alcohol used in the saponification of the purified butter fat was refluxed over sodium hydroxide and redistilled to remove acidic products. The mixed acids from 399 gm. of fat were steam-distilled for 6 hours, the aqueous distillate being extracted with ether and dried over neutral anhydrous sodium sulfate. The steam-volatile acids were fractionally distilled after recovery of the ether. The ether-extracted aqueous solution, the recovered ether, and the sodium sulfate were acidic. This acidity was determined by titration with standard alkali and calculated as butyric acid. Acids non-volatile in steam were resolved into "solid" and "liquid" portions by a modified Twitchell lead salt separation (29) in which 10 cc. of 95 per cent alcohol (containing 1.5 per cent of acetic acid) per gm. of mixed fatty acids were used for the precipitation and recrystallization of the insoluble lead

salts (2, 8). The neutral methyl esters of each group of acids were fractionally distilled. These details of analysis are summarized in Tables I to III. Methods and assumptions employed in

TABLE I  
*Preliminary Resolution of Mixed Butter Fatty Acids by Steam Distillation and Lead Salt Separation*

	gm.	per cent	Corresponding methyl esters	
			Saponification equivalent	Iodine value
Volatile acids.....	17.45	4.7		
Non-volatile acids.....	356.2	95.3		
"Solid" acids.....	181.0	48.5	271.2	7.7
"Liquid" ".....	175.2	46.8	264.0	74.2

TABLE II  
*Fractional Distillation of Steam-Volatile Acids (735 Mm.)*  
Acidity (as butyric acid) in ether-extracted aqueous solution, 0.16 gm.; recovered ether, 0.04 gm.; sodium sulfate (used for drying), 0.01 gm.

Distillate fraction No.	Boiling point	Amount	Saponification equivalent	Iodine value
	°C.	gm.		
V1	35	0.25	*	
V2	76-78	1.17	*	
V3	78-79	2.34	*	
V4	82-90	1.48	*	
V5	155-162	2.11	132.6	
V6	162	3.76	90.1	
V7	162-164	3.82	89.7	
V8	164-183	1.13	93.7	
V9	183	2.86	99.6	0.0
V10	Residue	4.34	134.1	9.1

\* Neutral to phenolphthalein.

calculating the component acids (Table IV) have been cited previously for the non-volatile acids (17, 20); calculation of the steam-volatile acids is straightforward.

The presence of unsaturated esters of low molecular weight was

TABLE III  
*Fractional Distillation of Methyl Esters of Non-Volatile Acids*

Fraction No.	Amount	Saponification equivalent	Iodine value (Wijs)
Methyl esters of "solid" acids			
	<i>gm.</i>		
S1	2.42	225.5	2.5
S2	3.30	238.7	0.6
S3	4.00	248.7	0.8
S4	7.52	249.9	0.7
S5	7.57	261.1	0.9
S6	8.39	266.1	1.1
S7	9.09	268.2	0.6
S8	9.01	270.2	2.4
S9	8.41	278.5	10.2
S10	18.90	295.3	21.7
S11	4.75	310.2	25.4
	83.36		
Methyl esters of "liquid" acids			
L1	0.01*		
L2	1.48	128.3	1.1
L3	2.39	153.9	0.7
L4	1.60	172.7	6.2
L5	3.94	185.5	12.0
L6	5.40	190.5	12.0
L7	3.06	205.9	11.1
L8	2.25	213.6	13.9
L9	3.58	215.1	17.6
L10	3.15	227.0	20.0
L11	5.82	240.2	31.9
L12	3.73	257.1	56.3
L13	8.34	260.2	57.0
L14	4.94	253.4	41.1
L15	6.10	267.0	60.9
L16	11.86	288.7	94.8
L17	4.00	294.3	95.2
L18	66.68	295.6	96.6
L19	9.13	296.1	105.2
L20	1.88	298.6	112.7
L21	6.84	357.7	103.1
	156.18	301.8†	

\* Low boiling, non-fatty material.

† Esters freed from unsaponifiable matter.

again indicated in these analyses. In order to obtain further confirmation of their presence we have chosen to oxidize completely several fractions which appeared to contain as high a concentrate of saturated and unsaturated esters of the same molecular weight as it was possible to obtain by the fractional distillation of such relatively small amounts of material. Bromination of larger amounts of similar fractions followed by a fractionation (3, 26) is a useful procedure for the isolation of these

TABLE IV  
*Component Fatty Acids of Cow's Milk Fat*

Acids	Volatile acids (4.7 per cent)	"Solid" acids (48.5 per cent)	"Liquid" acids (46.8 per cent)	Total	Per cent	
					Weight	Molar
Butyric.....	3.01		0.02	3.03	3.0	8.1
n-Hexanoic.....	0.92		0.47	1.39	1.4	2.8
n-Octanoic.....	0.70		0.81	1.51	1.5	2.5
n-Decanoic.....			2.68	2.68	2.7	3.7
Lauric.....		0.93	2.78	3.71	3.7	4.4
Myristic.....		9.35	2.66	12.01	12.1	12.5
Palmitic.....		23.15	2.02	25.17	25.3	23.2
Stearic.....		9.17		9.17	9.2	7.6
As arachidic.....		1.32		1.32	1.3	1.0
$\Delta^{9,10}$ -Decenoic.....	0.07*		0.25	0.32	0.3	0.4
$\Delta^{9,10}$ -Dodecenoic.....		0.03	0.39	0.42	0.4	0.9
$\Delta^{9,10}$ -Tetradecenoic.....			1.58	1.58	1.6	1.7
$\Delta^{9,10}$ -Hexadecenoic.....		0.20	3.76	3.96	4.0	3.7
Oleic.....		4.35	25.19	29.54	29.6	24.8
$\Delta^{9,10,12,13}$ -Octadecadienoic.....			3.57	3.57	3.6	2.9
As arachidonic.....			0.28	0.28	0.3	0.2
Unsaponifiable.....			0.34	0.34		

\* If the unsaturated acid present in the volatile acids is calculated as oleic acid, the amount of the latter is 0.08 per cent.

unsaturated esters. We prefer, however, the isolation of the saturated esters and determination of their saponification equivalent in these cases.

Oxidation of the unsaturated esters with permanganate in acetone was readily accomplished by the method of Armstrong and Hilditch (1). The esters were dissolved in boiling acetone and finely divided permanganate was added gradually. (The iodine value of ester fractions can be reduced to less than 1.0 from as high as 70 in one operation by use of 10 gm. of permanganate



and 20 cc. of acetone per gm. of esters.) After the material had been refluxed for 2 to 3 hours, the acetone was removed, manganese oxides were decolorized with sulfur dioxide, and the unchanged saturated esters were extracted with ether, which was washed with a 10 per cent aqueous potassium carbonate solution to remove acidic products formed during the oxidation. These washings were acidified and exhaustively steam-distilled. Saponification equivalents of the dried ether extract of these distillates were determined when possible. Dibasic acids formed during the oxidations were obtained after saponification and reacidification of the residues from the steam distillation. Ester Fractions L5, L8-9, L11, and L15 were thus examined.

TABLE V

*Saturated Esters Present in Ester Fractions Containing Decenoic, Do-, Tetra-, and Hexadecenoic Acids*

Fraction No.	Original esters			Saturated esters	
	Amount	Saponification equivalent	Iodine value	Amount	Saponification equivalent
	<i>gm.</i>			<i>gm.</i>	
L5	3.68	185.5	12.0	2.45	186.4
L8-9	4.29	214.4	15.6	3.10	214.6
L11	5.59	240.2	31.9	3.72	241.4
L15	5.65	267.0	60.9		268.1

As may be observed in Table V, the saturated esters obtained were found to have practically the same saponification equivalent as determined for the original esters. The acids from these saturated esters were identified as *n*-decanoic, lauric, myristic, and palmitic. It is obvious from these data that the following unsaturated esters were present.

Fraction No.	Methyl ester	Theoretical saponification equivalent
L5	Decenoic	184.2
L8-9	Dodecenoic	212.2
L11	Tetradecenoic	240.2
L15	Hexadecenoic	268.3

Examination of the potassium carbonate washings clearly revealed the similar structure of the C<sub>10</sub>, C<sub>12</sub>, C<sub>14</sub>, and C<sub>16</sub> unsatu-

rated acids present in the original ester fractions. Azelaic acid,  $C_9H_{18}O_4$ , m.p.  $104^\circ$ , was identified in 65 to 75 per cent of the theoretical amounts in each case. The saponification equivalent of the volatile acids produced by oxidation of the  $C_{14}$  unsaturated esters was 103.1 ( $C_8H_{16}O_2 = 102.1$ ); from the  $C_{16}$  unsaturated ester, volatile acids with a saponification equivalent of 132.1 were obtained ( $C_7H_{14}O_2 = 130.1$ ). The volatile acids formed by oxidation of the  $C_{10}$  esters were not examined and too small an amount was obtained in the case of the  $C_{12}$  ester oxidation for molecular weight determination. In the case of each unsaturated acid, therefore, the position of the ethenoid linkage is the same with respect to the carboxyl group ( $\Delta^{9,10}$ ). With the addition of oleic and the octadecadienoic acids, which are mentioned later, it is interesting at this point to record the following structures and molecular proportions for the unsaturated acids (except arachidonic) of cow's milk fat.

		Per cent (molar)
Decenoic.....	$CH_2:CH(CH_2)_7COOH$	0.4
Dodecenoic.....	$CH_3CH_2CH:CH(CH_2)_7COOH$	0.9
Tetradecenoic.....	$CH_3(CH_2)_2CH:CH(CH_2)_7COOH$	1.7
Hexadecenoic.....	$CH_3(CH_2)_4CH:CH(CH_2)_7COOH$	3.7
Octadecenoic (oleic).....	$CH_3(CH_2)_7CH:CH(CH_2)_7COOH$	24.8
Octadecadienoic....	$CH_3(CH_2)_4CH:CH\cdot CH_2\cdot CH:CH(CH_2)_7COOH$	2.9

The occurrence of minor proportions of monoethenoid acids down to, but not below,  $C_{10}H_{18}O_2$ , in progressively smaller proportions, and the circumstance, which we have now demonstrated, that in all cases the ethenoid linkage occupies the  $\Delta^{9,10}$  position is very suggestive in connection with the hypothesis (18, 22) that the lower fatty acids in milk fats are produced by combined oxidation and reduction of oleic groups which already exist in the form of glycerides. The minor unsaturated components of milk fat may represent fragments of transformed oleoglycerides which have escaped complete saturation to lower saturated glycerides.

Bromination of the  $C_{18}$  unsaturated acids present in the ester Fraction L18 (Table III) and the  $C_{18-20}$  acids present in Fraction L20 has borne out again the experience of previous investigators who have studied these acids from milk fat. Very small amounts of ether- and petroleum ether-insoluble bromides were obtained in each case. These amounted to 0.1 per cent and 0.6 per cent of the total weight of bromo-additive products formed from the acids

in Fraction L18 and 1.6 per cent and 0.9 per cent in the case of Fraction L20. By far the largest proportion of the bromo-additive products in each case was soluble in ether and petroleum ether at 0°, being 99.3 per cent for Fraction L18 and 97.5 per cent for Fraction L20. The tetrabromostearic acid, m.p. 114°, characteristic of seed fat linoleic acid could not be identified, however, among the petroleum ether-insoluble products, which melted at 170° in each case. Nor could the liquid ether- and petroleum ether-soluble products, which contained 40.9 per cent and 41.4 per cent of Br (decidedly above that for dibromostearic acid, 36.2 per cent Br), be crystallized. The ether-insoluble bromides from Fraction L18 melted at 185°; those from Fraction L20 melted at 215–217° (with decomposition). This fact, together with the increased amount, however low, of ether-insoluble bromides in Fraction L20 may indicate the presence of arachidonic acid (m.p. octabromide, 220°).

This difficulty in the identification of the octadecadienoic acid present in cow's milk fat by bromination is, curiously enough, not wholly paralleled in its mild oxidation to the characteristic tetrahydroxystearic acids, m.p. 155° and 173°, which ordinarily result on similar oxidation of seed fat linoleic acid. We readily obtained both these derivatives using the essentials of the method of Lapworth and Mottram (24) as described by Hilditch and Longenecker (20). Here, however, as in the case of the C<sub>18</sub> unsaturated acids of ox depot fat and likewise the C<sub>18</sub> acid of milk fat oxidized by the modified Hazura process (14, 11), the yields of tetrahydroxy acids were only 10 to 12 per cent of the theoretical, when the original mixture of acids is calculated as mono- and diethenoid, whereas with ordinary seed fat linoleic acid the yields are definitely higher, about 40 per cent (10). The yields of dihydroxystearic acid, m.p. 132°, obtained in these oxidations were 80 to 90 per cent of the theoretical.

The analysis of this sample of butter fat with an electrically heated and packed column is in good agreement with previous analyses from this laboratory in which the ester fractionations were accomplished with simpler fractionation equipment (Willstätter bulb). Thus the figures of Hilditch and Paul (21) approximate our results very closely, irrespective of individual variation due to feeding, climate, etc. Only two notable differences in our

data are observed: (1) the figure for myristic acid (12.5 moles per cent) is slightly higher than is usually recorded and (2) the amount of oleic acid is decreased in proportion to the amounts of lower molecular unsaturated acids present; the proportions of the other acids remain practically unaffected.

#### SUMMARY

With an electrically heated and packed column for the ester fractionations in the analysis of cow's milk fat, it has been possible to obtain fractions with very high proportions of unsaturated esters of low molecular weight. The presence of decenoic, do-, tetra-, and hexadecenoic acids has thus been demonstrated; the ethenoid linkage in each unsaturated acid occupied the  $\Delta^{9,10}$  position in the carbon chain. Bromination and mild oxidation with alkaline permanganate of the  $C_{18}$  unsaturated acids confirm earlier conclusions regarding the presence of only oleic acid and an octadecadienoic acid. The presence of arachidonic acid was indicated by a study of its bromo-additive products. Saturated acids which have been isolated and identified were octanoic, *n*-decanoic, lauric, myristic, palmitic, and stearic. The analysis for the component fatty acids of milk fat, one of the most complex of all fats, is apparently accomplished with similar results when either an electrically heated and packed column for the fractional distillation of ester mixtures, or some simpler apparatus (Willstätter bulb), is employed.

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## **A METHOD FOR THE PREPARATION OF POSTHEMO- LYTIC RESIDUE OR STROMA OF ERYTHROCYTES\***

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A variety of hematological and physicochemical observations have been employed in elucidating the structure of the red blood cell (2, 3). Although these aspects have been studied, comparatively few investigations have been reported concerning the chemical make-up of the erythrocyte framework. Knowledge of the chemical composition of all parts of the red blood cell is essential in accurately defining its structure and functions and in understanding the abnormalities that occur in various types of anemia (4, 5).

The analogy of the erythrocyte to a membrane-covered balloon containing hemoglobin in solution was formulated by observations on the release of hemoglobin from the erythrocyte and the remaining cell shadows or "ghosts" upon hemolysis (6, 7). Hemolytic and microscopic studies have confirmed the presence of an outside capsule (3, 8). However, an internal structural beamwork, as well as an enclosing membrane, has been suggested as necessary for the erythrocyte to maintain its characteristic "biconcave" shape (2, 9, 10).

Methods for isolating the insoluble part of the red cell which remains after hemolysis, designated as the "stroma" of the erythrocyte, can be found throughout the literature of the past century. This term was introduced by Rollett (9) in 1870 when he postulated a spongy framework in the interior of the red blood cell. Whether the stroma forms a cell envelope, an internal

\* Presented before the American Society of Biological Chemists at its Thirtieth annual meeting at Washington, D. C., March 25-28, 1936 (1).

network in the meshes of which hemoglobin is held, or a combination of both features, remains to be determined. Few attempts have been directed towards the purification of stroma preparations and a study of the chemical composition (11-16).

In a series of investigations on the structure of the red blood cell, posthemolytic residues from the erythrocytes of various animals have been prepared. Various procedures have been studied and one developed which yields a sufficient residue with little hemoglobin contamination. A detailed discussion of the lipid composition (17) and amino acid distribution of the protein of the stroma in relation to cellular structure will be presented in subsequent reports.

The blood samples,<sup>1</sup> obtained from abattoirs at the time of slaughter, were defibrinated by whipping, cooled, and used at once. The human blood was obtained by venous puncture, citrated, and chilled. The erythrocytes were separated by ordinary centrifuging and then washed with cold physiological sodium chloride solution until all of the plasma, leucocytes, and platelets had been removed. After hemolysis, centrifugation, and washings the residues were dried *in vacuo* at 35° and powdered for detailed analyses. Efforts were made to avoid as much denaturation as possible by working rapidly and chilling the cells, hemolysates, and wash solutions.

The presence of organic iron in the dried residues was considered *prima facie* evidence for the presence of hemoglobin and was used as one of the criteria of purity, although it is possible that iron may be combined organically in forms other than hemoglobin in the posthemolytic residue (18, 19). The total iron analyses were made on ashed samples by a method developed in this laboratory (20). The inorganic iron, much of which was picked up from the metal bowl of the centrifuge in the course of preparation, was determined by the method of Kohler, Elvehjem, and Hart (21). The organic iron content was then calculated by subtracting the inorganic from the total iron present.

The uniformity of the protein and lipid composition served as additional evidence of purity of the stroma sample. The lipid

<sup>1</sup> The authors wish to thank Mr. Harvey Merker and Dr. L. C. Clark, of Parke, Davis and Company, Detroit, for their cooperation in securing the equine blood.

and nitrogen analyses were made by the gasometric methods of Kirk, Page, and Van Slyke (22, 23) with certain modifications (24-26).

Preparations were made by the method of Haurowitz and Sládek (12) with toluene as the hemolytic agent, by Chou's method (13) with ether, by freezing-thawing hemolysis,<sup>2</sup> and by buffering to pH 5.5 with 0.1 N HCl (14, 16). None of these methods resulted in a satisfactory product being recovered. In an effort to recover larger amounts of the residue lost by the freezing-thawing method, salting-out was attempted by addition of ammonium sulfate to the effluent of the third washing. The resulting product was purer but altered in composition.

None of the foregoing methods produced desirable results and considerable irregularity in hemoglobin contamination and chemical composition was manifest, even in preparations by identical methods from the same blood sample (Table I), indicating that the hemoglobin may be held, in part at least, by adsorption and that the final product may be changed through the use of a strong acid. A buffering solution of a weak acid mixture of sodium citrate and 0.1 N sodium hydroxide<sup>3</sup> in the adjustment of the pH was studied.

A liter of washed bovine erythrocytes was hemolyzed in 20 volumes of distilled water and chilled overnight. The mixture was then passed through the supercentrifuge at 40,000 R.P.M.<sup>4</sup> four successive times. The stroma was repeatedly washed by prolonged stirring in distilled water, centrifuged until the washings were free from color, and then dried.

<sup>2</sup> We are grateful to Dr. Eric Ponder for suggesting the freezing-thawing method of preparing stroma.

<sup>3</sup> The sodium citrate solution was made by dissolving 21.008 gm. of citric acid in 200 cc. of carbonate-free N sodium hydroxide solution and diluting to a liter. Sørensen's directions for buffer mixtures of sodium citrate and 0.1 N sodium hydroxide were followed ((27) p. 112). For hemolysis of the erythrocytes this buffer solution was diluted 1:15 and adjusted to pH 5.5.

<sup>4</sup> A steam turbine drive, medical model, laboratory type Sharples supercentrifuge was purchased from the Sharples Specialty Company, 23rd and Westmoreland Streets, Philadelphia. (This centrifuge can be arranged to deposit a precipitate on the sides of the bowl or separate fluid layers of different densities.)



# 510 Posthemolytic Residue of Erythrocytes

TABLE I

*Comparison of Methods for Preparation of Posthemolytic Residues of Erythrocytes*

Sample	Sample No.	Volume of erythrocytes	Yield per 100 cc. erythrocytes	Color of dried preparation	Total iron	Inorganic iron	Organic iron	Hemoglobin	Protein*	Lipid	Ash	Total†
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## Hemolysis with toluene

		cc.	gm.		per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Bovine	4	400	0.24	Red-brown	0.35	0.13	0.22	64		8	5	
Sheep	7	400	0.36	Tan	0.16	0.12	0.04	13	61	14	2	90

## Hemolysis with ether

Bovine	5	400	0.42	Brown	0.16	0.04	0.12	37	23	18	7	85
Sheep	8	375	0.38	Tan	0.08	0.04	0.04	12	42	29	6	89

## Hemolysis by freezing and thawing

Bovine	10	400	0.10	Red-brown	0.29	0.06	0.23	69		18		
"	14	400	0.11	Brown	0.12	0.03	0.09	27	33	23	5	88
"	16	550	0.11	Light brown	0.06	0.02	0.04	11	43	24	4	82
"	17	550	0.07	Brown	0.10	0.04	0.06	19	29	27	8	88
Sheep	13	300	0.21	Light red	0.24					17		

## Separation with ammonium sulfate

Bovine (residue from effluent of Sample 14)	15			Gray	0.05	0.02	0.03	8	74	15	3	100
Bovine	18	500	0.15	Red-brown	0.20	0.04	0.16	48	7	8	23	86

## Hemolysis with 10 volumes of water, pH adjusted to 5.5 with 0.1 N HCl

Bovine	2	400	0.48	Light brown	0.05	0.02	0.03	10	45	25	8	88
Sheep	9	200	0.59	" red	0.11	0.06	0.05	16	55	16	3	90
"	11	400	1.05	" "	0.10	0.04	0.06	18	47	25	2	92
Equine	6	500	0.45	Gray-brown	0.07	0.05	0.02	8	80		3	

TABLE I—*Concluded*

Sample	Sample No.	Volume of erythrocytes	Yield per 100 cc. erythrocytes	Color of dried preparation	Total iron	Inorganic iron	Organic iron	Hemoglobin	Protein*	Lipid	Ash	Total†
Before and after buffering to pH 5.5 with sodium citrate buffer												
		cc.	gm.		per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Bovine (hemolyzed with buffer)	22		0.19	Brown	0.10	0.03	0.07	22	38	26	4	90
Bovine (unbuffered)	29	1000	0.22	Light brown	0.09	0.06	0.03	11	54	22	5	92
Bovine (residue from effluent of Sample 29)	30		0.61	Gray-white	0.06	0.04	0.02	5	65	19	3	92

\* Total protein minus hemoglobin.

† Sum of hemoglobin, protein,\* lipid, and ash.

The effluent material from this hemolysate was buffered to a pH of 5.5 with sodium citrate solution, chilled, and centrifuged as outlined above, but the residue was washed with the buffer solution in place of distilled water.

Another residue was prepared in which the erythrocytes were hemolyzed in 20 volumes of the sodium citrate solution, producing a hemolysate at pH 6.5. The precipitate was separated in the supercentrifuge, without further buffering, and was repeatedly washed in the citrate solution with prolonged stirring.

The use of the sodium citrate buffer solution (pH 5.5) as a hemolytic and washing agent, when the laked erythrocyte solution is not adjusted to a pH of 5.5, gave results similar to the use of distilled water alone (Sample 29). The total lipid and protein contents of the final product (Sample 22) were slightly higher, but a much larger proportion of the protein consisted of hemoglobin. Buffering the effluent material (Sample 29) after the preliminary centrifugation tripled the quantity of stroma recovered (Sample 30). The hemoglobin contamination of this additional material was only 5 per cent as compared to 11 per cent in the unbuffered

## 512 Posthemolytic Residue of Erythrocytes

preparation, whereas the non-hemoglobin protein contents were 65 and 54 per cent respectively. It seemed that the two preparations were similar and that the material precipitated by the buffer solution represented the posthemolytic residue in a purer form.

In view of the latter observation, a series of stroma preparations was made by hemolysis, adjusting to pH 5.5, and washing, citrate buffer solution being used throughout. These were sus-

TABLE II  
*Posthemolytic Residues of Erythrocytes Prepared by Sodium Citrate Buffer Method*

Sample	Sample No.	Volume of erythrocytes	Yield per 100 cc. erythrocytes	Color of dried preparation	Total iron	Inorganic iron	Organic iron	Hemoglobin	Protein*	Lipid	Ash	Total†
		cc.	gm.		per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Bovine	20	1300	0.31	Gray-white	0.04	0.02	0.02	6	57	25	3	91
	21	1200	0.59	"	0.04	0.02	0.02	6	59	26	2	93
	24	800	0.84	"	0.06	0.05	0.01	2		26	3	
	34			Light tan	0.05	0.02	0.03	7	56			
Sheep	26	750	0.67	" "	0.13	0.12	0.01	2	68	24	3	97
Equine	22	600	1.26	" pink	0.05	0.02	0.03	10	53	20	2	85
Human	25	220	0.72	Dark red	0.28	0.17	0.11	34	39	10	5	88
	27	120	0.56	Red-brown	0.22	0.16	0.06	14	59	11	5	89
	32	140	1.07	"	0.11	0.04	0.07	20	50	12	5	87
Avian												
Chicken	28	400	5.24	"	0.09	0.09	0.00	1	91	2	2	96
"	35			"	0.04	0.02	0.02	7	91	6	1	105
Turkey	33	400	6.29	Dark brown	0.04	0.02	0.02	7	86	2	3	98

\* Total protein minus hemoglobin.

† Sum of hemoglobin, protein,\* lipid, and ash.

pending in 4 liters of cold sodium citrate buffer solution (pH 5.5), packed in a container of ice, and stirred for 6 to 8 hours with an electric stirrer, after which the stroma was again separated from the wash solution by centrifuging at 40,000 R.P.M. These processes were repeated four to six times and the final product dried and powdered for subsequent analysis.

Stroma samples from bovine, horse, sheep, human, chicken, and turkey erythrocytes were prepared by this method. The yields recorded in Table II, varying from 0.3 to 1.3 gm. of dried stroma

per 100 cc. of erythrocytes, illustrate the superiority of this method. In addition this method yielded as much as 5 to 6 gm. of stroma from a similar quantity of avian cells. Furthermore, the product was purer, containing as little as 2 per cent and not more than 10 per cent hemoglobin, except in the case of human stroma which had 14 to 34 per cent hemoglobin.

The posthemolytic residues of the red blood cells exhibited definite variations in physical properties among the species studied. The color of the dried stroma differed from a gray-white to dark brown in the case of bovine and turkey red blood cells respectively, and was not related to the hemoglobin contained therein. The bovine and sheep stromata formed fine particles which required high speed centrifugation to separate, whereas those of the horse, chicken, and human erythrocytes were coarse and settled readily on standing. The ease with which the hemoglobin could be washed out also varied, being most difficult in the preparation of human stroma, thus suggesting the presence of organic iron-containing compounds other than hemoglobin. The posthemolytic products also demonstrated differences in chemical composition which have been discussed (17) in respect to the hematological and physicochemical properties of the erythrocytes of various species.

#### SUMMARY

A comparison has been made of various procedures for the preparation of stroma involving hemolysis of erythrocytes with toluene, ether, water, freezing and thawing, and removal of the stroma by a supercentrifuge at 40,000 R.P.M. In addition, the effect of salting-out with ammonium sulfate, the adjustment of the pH with 0.1 N hydrochloric acid and with a sodium citrate buffer solution upon the yield, composition, and purity of the products were investigated. A procedure was finally developed based upon hemolysis of the erythrocytes and prolonged washing of the stromata with large volumes of sodium citrate buffer at pH 5.5.

Posthemolytic residues from bovine, sheep, equine, avian, and human erythrocytes have been prepared with comparatively little hemoglobin contamination and in sufficient quantities for detailed lipid and protein analyses.

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## THE LIPID DISTRIBUTION OF POSTHEMOLYTIC RESIDUE OR STROMA OF ERYTHROCYTES\*

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The physicochemical properties of the erythrocyte surface have been well defined by a variety of observations. Conductivity experiments have confirmed the presence, around the erythrocyte, of a relatively non-conducting envelope (2) which, on the basis of electrical capacity measurements, is approximately  $0.003 \mu$  thick (3), or within the magnitude of molecular dimensions. That it is a membrane which can be folded, stretched between microdissection needles, and even drawn out into long processes has been shown by unique hemolytic (4, 5) and microscopic studies (6). The erythrocytes of various species of animals exhibit characteristic differences in hematologic (4, 5, 7), hemolytic (4, 5), cataphoretic (8), and permeable (9, 10) properties, indicating essential differences in their surface composition and structure.

Knowledge concerning the chemical nature of the erythrocyte membrane is incomplete and postulations have differed as to whether it consists of lipids, protein, or a protein-lipid complex. The erythrocyte surface demonstrates definite protein characteristics: the gel-like behavior and elastic properties of the red cell membrane (11) are similar to those of gels or films formed by protein made up of thread-like micelles (12), and its permeability to certain substances appears dependent upon the presence of a porous or sieve-like structure (10, 13). Contrariwise, the extreme permeability of erythrocytes to fat-soluble substances (10, 14) and the absence of a reversal in ionic permeability with changes

\* Presented before the American Society of Biological Chemists at its Thirtieth annual meeting at Washington, D. C., March 25-28, 1936 (1).

in pH (15) indicate that the surface consists of a homogeneous lipid layer. Surface area measurements on unimolecular films of lipids extracted from erythrocytes have led Gorter and coworkers (16) to suggest that there is just sufficient lipid present to form a membrane with a thickness of 2 molecules. Since the lipids of the red blood cell consist chiefly of phospholipids and cholesterol (17), it is significant that phospholipids have been shown to form coacervates which, with sensitizers such as cholesterol, become oriented in stable bimolecular films (18).

The diversified evidence concerning the chemical structure of the red blood cell, however, has been reconciled by postulating a mosaic-like membrane containing both protein and lipid (6, 8, 10, 13, 19). The cataphoretic velocities of erythrocytes (8) and the selective permeability for a variety of substances (10, 13) may be explained on the basis of coexisting protein and lipid areas on the surface. Furthermore, optical studies of erythrocyte ghosts have demonstrated the presence of layers of both thread-like protein and interspersed lipid micelles with their optical axes oriented tangentially and radially, respectively (20).

The development of methods for isolating a posthemolytic residue from erythrocytes has opened new avenues for the investigation of the structure of the red blood cell (21). Since isolated stroma has been shown to exhibit essentially the same physico-chemical properties as those of the intact cell (cataphoretic mobility (8, 22), agglutination, and serological reactions (23, 24)), it is evident that these residue substances play a vital rôle in the surface phenomena of the erythrocyte at least. Whether the stroma forms the covering membrane, an internal network, or a combination of both, remains to be determined.

Posthemolytic residue samples, comparatively free of hemoglobin, have been prepared from erythrocytes of various animals (21). These preparations have been analyzed for total protein, ash, and lipids. The observations recorded herein have included the free and ester cholesterol, neutral fat, and phospholipids, together with results obtained in the estimation of the individual phospholipids—lecithin, cephalin, and sphingomyelin. The stroma analyses were accompanied by similar lipid studies of the intact erythrocytes together with observations on red cell count, hemoglobin, hematocrit, diameter, specific gravity, water content, and calculations of the corpuscular dimensions and composition.

Amino acid analyses of the stroma protein are in progress and will be reported later.

### Methods

The stroma samples were prepared by the method described and discussed in the preceding paper (21). The methods for the hematological observations, water content, specific gravity, and corpuscular measurements<sup>1</sup> on the erythrocytes, as well as the method of separation of erythrocytes for analysis, have been discussed in a preceding report from this laboratory (17). Nitrogen was determined on 15 mg. samples of dried stroma by the micro-Kjeldahl gasometric technique of Van Slyke ((25) p. 353, (26)).

50 mg. of the dried powdered stroma and 3 to 4 gm. of packed red blood cells were transferred to 100 cc. volumetric flasks and brought to boiling in a 3:1 mixture of alcohol-ether, after which they were set aside for several days to extract at room temperature. Total cholesterol and phosphorus determinations were made on the alcohol-ether extracts; total phosphorus, carbon, and free cholesterol determinations, on the petroleum ether extracts, with the gasometric microlipid methods of Kirk, Page, and Van Slyke (27, 28).

Van Slyke's procedure for the evaporation of the alcohol-ether and reextraction with petroleum ether was employed in some of the earlier analyses. Since work from this (29) and other laboratories (30, 31) has shown that this procedure often results in low recoveries of phospholipids, the evaporation of alcohol-ether extracts *in vacuo*, in an atmosphere of nitrogen as recommended by Man (30) with which recovery is practically complete, was adopted for the remaining samples and repeated on a number of the earlier preparations.<sup>2</sup> The choline in hydrolyzed phospholipids of erythrocytes and stroma was determined as a means of

<sup>1</sup> It was necessary to extend the calibration of Bock's erythrocytometer. This was done by measuring the distance in cm. of each calibration mark above the frosted glass plate. An equation was fitted to the resulting curve from which readings falling outside the calibration scale on the instrument were calculated. The diameter  $\mu = -0.0184 \text{ cm.}^2 - 0.0307 \text{ cm.} + 9.2614$ . We appreciate the assistance of Dr. Harriett J. Kelly in computing this equation.

<sup>2</sup> Details of the procedure will appear in a following paper (32).



differentiating the individual phospholipids (32). A procedure was developed for securing stroma, involving hemolysis with a sodium citrate buffer solution at pH 5.5 followed by repeated washings and prolonged stirrings with the same solution (21).

#### DISCUSSION

The lipid distribution of the posthemolytic residue samples as recorded herein may serve as one of the criteria for the preparation of chemically unaltered stroma, representative of that in the intact cell, and may furnish information on cellular structure.

There was considerable variation in the lipid content of the residues, particularly those prepared by the toluene method. These residues contained much less lipid than any of the other samples (Table I). The finding that stroma isolated from erythrocytes by the ether hemolysis procedure contained as much lipid as samples separated by physicochemical means furnished additional evidence of a firm lipid-protein complex in the stroma (6, 8, 10, 13, 19). Ether is known to be ineffective in extracting certain lipid-protein mixtures which can be separated only after preliminary extraction with alcohol and may even require partial hydrolysis of the protein (33, 34). The toluene, ether, and the other preliminary procedures were discarded because of the low yields, high hemoglobin contamination, and irregularities in chemical composition.

The separation of the stroma by the citrate buffer method was adopted for the study of the composition of the red blood cell, since it resulted in larger yields of a purer product which was more uniform in composition (21). The lipid content of samples prepared by this procedure was strikingly constant for erythrocytes of each species, as shown in Table II. The dried stroma of bovine, sheep, and horse erythrocytes was similar in lipid content, with an average of 25, 24, and 20 per cent respectively, whereas human red cell stroma contained 11 per cent and that prepared from the avian erythrocytes contained appreciably less, averaging 3 per cent. The intact erythrocytes of these species demonstrated differences in lipid content which did not appear to be related to the amount in the corresponding stroma preparations. Average total lipid values for sheep, avian, human, and bovine red blood cells were 595, 550, 424, and 375 mg. per 100 gm. of packed red cells, respectively (Table III).

**TABLE I**  
*Lipid and Protein Composition of Posthemolytic Residue Prepared by Various Methods*

Sample	Sample No.	Per cent composition					Per cent distribution of total lipids				Cephalin,† per cent of phospholipid	Protein* to lipid ratio	
		Protein*	Total lipid	Phospholipid	Cholesterol		Neutral fat	Phospholipid	Cholesterol				Neutral fat
					Free	Esters			Free	Esters			
Hemolysis with toluene													
Bovine	4		7.5‡	1.8	2.2	0.0	3.5	24	30	0	46		
Sheep	7	60.5	13.9‡	6.9	4.2	0.9	1.9	50	30	6	14		4.4
Hemolysis with ether													
Bovine	5	23.3	18.0	10.5	4.2	1.0	2.3	58	23	6	13	50	1.3
Sheep	8	42.0	29.3	16.5	6.8	0.7	5.3	56	23	3	18	62	1.4
"	12	46.2	28.8‡	13.6	4.8	8.4	1.9	47	17	29	7	60	1.6
Hemolysis by freezing and thawing													
Bovine	10		18.3‡	9.9	4.8	0.3	3.4	54	26	2	18		
"	14	32.7	23.2	15.6	5.9	1.7	0.2	67	25	7	1		1.4
"	16	42.4	24.1‡	12.3	3.6	8.2	0.0	51	15	34	0		1.8
"	17	28.9	26.8‡	10.9	7.2	2.6	6.0	41	27	10	22		1.1
Sheep	13		17.4‡	4.9	9.2	0.0	3.3	28	53	0	19		
Hemolysis with 10 volumes of water, pH adjusted to 5.5 with 0.1 N HCl													
Bovine	2	44.8	25.2	13.7	5.2	1.9	4.4	54	21	8	17	55	1.8
Sheep	9	54.7	15.8	8.7	3.7	0.0	3.4	55	23	0	22	49	3.5
"	11	46.6	24.6	13.5	6.8	0.2	4.1	55	28	1	16	63	1.9
Separation with ammonium sulfate													
Bovine (residue from effluent of Sample 14)	15	74.3	14.6‡	9.3	3.0	1.0	1.4	64	20	7	9		5.1
Bovine	18	6.6	8.1‡	1.7	2.0	1.5	2.9	21	25	18	36		0.8
Before and after buffering to pH 5.5 with sodium citrate buffer													
Bovine (hemolyzed with buffer)	22	38.0	25.7‡	12.8	5.9	1.4	5.6	50	23	5	22		1.5
Bovine (unbuffered)	29	54.4	22.0	14.6	5.0	0.0	2.4	66	23	0	11	54	2.5
Bovine	30	64.6	18.7	13.5	4.4	0.3	0.5	72	24	1	3	58	3.5

\* Total protein minus hemoglobin.

† Calculated from the choline determinations.

‡ Lipid determined on alcohol-ether extracts evaporated in air.

The erythrocytes of the various species differed in their hematological characteristics, particularly in their hemoglobin content, size, and weight, as shown in Table IV. The red blood cells of the sheep and ox were small, weighing approximately 41 and 61 micromicrograms respectively, as compared to human and avian cells weighing 93 and 130 micromicrograms. Because 100 gm. of sheep cells contained approximately 2 and 3 times as many

TABLE II  
*Composition of Posthemolytic Residues Prepared by Sodium Citrate Buffer Method*

Sample	Sample No.	Per cent composition						Per cent distribution of total lipids				Cephalin, † per cent of phospholipid	Protein* to lipid ratio
		Protein*	Total lipid	Phospholipid	Cholesterol		Neutral fat	Phospholipid	Cholesterol		Neutral fat		
					Free	Esters			Free	Esters			
Bovine	20	56.6	25.4	16.3	7.2	0.7	1.2	64	28	3	5	67	2.2
"	21	58.5	25.5	15.9	7.0	1.1	1.5	62	28	4	6	58	2.3
"	24		25.9	15.9	6.5	0.0	3.5	61	25	0	14	49	
"	34	55.9		15.3									
Sheep	26	68.4	23.9	14.7	4.9	0.0	4.3	62	20	0	18	53	2.9
Equine	22	52.6	20.4	12.7	6.9	0.5	0.3	63	34	2	1	53	2.6
Human	25	39.4	10.4	6.0	2.6	0.0	1.8	58	25	0	17	75	3.8
"	27	59.0	11.1	7.3	1.8	0.8	1.3	66	16	6	12		5.3
"	32	50.2	12.0	8.6	2.4	0.6	0.4	72	20	5	3	49	4.2
Chicken	28	91.2	2.1	0.4	0.0	1.7	0.0	19	0	81	0		43.8
"	35	90.7	5.7	4.7	0.6	0.0	0.4	82	11	0	7	27	15.9
Turkey	33	86.1	1.9	1.6	0.3	0.0	0.0	84	16	0	0	41	45.3

\* Total protein minus hemoglobin.

† Calculated from the choline determinations.

erythrocytes as the same weight of human and avian cells, analyses expressed on a unit weight basis may obscure significant differences in the composition of the erythrocytes which may exist among the various species.

The lipid composition of an average single erythrocyte for each species has been calculated and the data given in Table III. On the basis of corpuscular composition the avian erythrocyte possessed appreciably more lipid than any of the other types with

TABLE III  
*Distribution of Lipids in Erythrocytes*

Sample	Erythrocytes, mg. per 100 gm. and per cent of total lipids										Corpuscle,† × 10 <sup>-12</sup> mg. per cell					
	Total lipids			Phospho-lipid*		Neutral fat		Choles-terol esters		Free choles-terol		Total lipids	Phospho-lipid*	Neutral fat	Cholesterol esters	Free choles-terol
	mg.	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent					
Bovine (average of 7) ..	375	207	55	40	11	35	9	93	25	228	126	24	21	57		
Sheep (average of 2) . . .	595	333	56	69	12	42	7	151	25	243	136	28	17	62		
Chicken (average of 2) . .	550	420	76	46	8	1	1	83	15	726	545	60	13	108		
Human† . . . . .	424	244	58	51	12	32	7	97	23	394	227	47	30	90		

\* Cephalin expressed as per cent of phospholipid as follows: bovine (average of 3) 57 per cent; chicken (average of 2) 33 per cent; human (average of 2) 59 per cent.

† Average values from the literature and studies in this laboratory (17).

‡ Represents the concentration in an average single red blood cell.

TABLE IV  
*Hematological Observations on Erythrocytes*

No. of studies.....	Cow 8	Sheep 2	Chicken 3	Human *
Red blood cells, millions per c.mm..	8.39	10.7	2.99	5.2
Hemoglobin				
Whole blood, gm. per 100 cc.....	13.4		10.3	14.0
Single cell, micromicrograms.....	16.3		34.4	29.0
Hematocrit, per cent.....	48.3	40.3	35.3	44.5
Corpuscular measurements				
Volume, cu.....	56.5	37.7	117.6	86.0
Weight, micromicrograms.....	61.1	40.8	129.7	93.0
Diameter, μ.....	5.85	5.28	12.10†	7.7
Thickness, ".....	2.1	1.9		2.0
Diameter to thickness ratio.....	2.8	2.8		4.1
Specific gravity.....	1.0874	1.0821	1.1067	1.0927
Water content, per cent weight.....	68	68	63	66

\* Average values taken from the literature and from studies in this laboratory (17).

† Value given by Ponder (7).

$726 \times 10^{-12}$  mg. per cell as compared to 394, 243, and  $228 \times 10^{-12}$  mg. for human, sheep, and bovine erythrocytes, respectively. These data show a relationship between the characteristic size and lipid content of the cell. On the other hand, the order of magnitude of erythrocyte lipid content for the different groups is diametrically reversed for that of the corresponding stroma preparations. For example, the intact avian red blood cell contained approximately 3 times as much lipid as that of the sheep, whereas the avian stroma had 3 per cent total lipid and the sheep 24 per cent.

Knowledge of the allocation of the various constituents within the intact erythrocyte is fundamental in ascertaining their rôle in cellular structure and function. Efforts were made to determine what proportion and which of the lipids are combined structurally as stroma, and which, if any, are loosely held or even mobile transport constituents within the cell and possibly liberated upon hemolysis.

Weighed samples of washed red blood cells were hemolyzed with 10 volumes of water and an aliquot portion of the fluid, including the suspended stroma, was withdrawn immediately for lipid analysis. The stroma was removed from the remaining hemolysate by adjustment of the pH to 5.5 with carbon dioxide gas, and by filtering through a special Jena glass filter of  $1.5 \mu$  porosity. An aliquot portion of stroma-free hemolysate for lipid analysis was made after careful microscopic examination in a dark-field showed the absence of cell shadows or ghosts.<sup>3</sup> With the exception of avian blood, 0 to 11 per cent of the total lipid represented in the intact erythrocyte was found in the stroma-free hemolysate and over half of this lipid usually consisted of neutral fat and cholesterol esters (see Table V). These observations indicate that the major portion of the red blood cell lipids is present structurally and does not play a rôle in the transport or metabolism of fat, which is in agreement with the conclusions drawn by Sinclair (35) from his studies of the metabolism of elaidic acid. Similar experiments on the erythrocytes of chicken gave conflicting results and in one case one-third of the lipid was found in the stroma-free hemolysate. These results point out the

<sup>3</sup> We wish to thank Dr. Pearl Lee of the Children's Hospital of Michigan, Detroit, for so kindly cooperating in making the dark-field examinations.

necessity and importance of knowing the exact location of each lipid fraction in the intact erythrocyte before its participation in structure and function can be defined.

It is of importance to determine whether the final posthemolytic residue contains the constituents originally present as stroma in

TABLE V

*Lipid Composition of Hemolyzed Erythrocytes with and without Stroma*  
The values are given in mg. per 100 gm. of erythrocytes.

Sample	Total lipid	Phospholipid	Cholesterol		Neutral fat
			Free	Esters	
Human erythrocytes					
With stroma.....	301	126	66	27	82
Without stroma.....	0	0	0	0	0
Bovine erythrocytes					
With stroma.....	380	218	92	60	10
Without stroma.....	49	0	0	38	10
With stroma.....	228	117	78	0	33
Without stroma.....	24	4	8	0	7
With stroma.....	438*	281†			
Without stroma.....	52*	18†			
With stroma.....	450*				
Without stroma.....	40*				
Sheep erythrocytes					
With stroma.....	318	203	114	1	0
Without stroma.....	0	0	0	0	0
With stroma.....	871*	463†			
Without stroma.....	94*	73†			
Chicken erythrocytes					
With stroma.....	510	364	102	0	44
Without stroma.....	25	10	15	0	0
With stroma.....	844*				
Without stroma.....	290*				

\* Calculated from total carbon of alcohol-ether extracts.

† Calculated from total phosphorus of alcohol-ether extracts.

the intact erythrocyte. This is particularly important in the case of stroma of the nucleated avian red blood cell where precipitation of appreciable amounts of nucleoprotein might explain the large yield of residue as well as an apparently lower percentage

of lipid.<sup>4</sup> Furthermore, the stability of the lipid-protein combination is of importance in securing posthemolytic residues representative of stroma as it exists in the cell. Investigations were made to determine whether any of the stroma lipids were lost in the process of preparation. Bovine and avian stromata were analyzed upon the first separation and after each washing and centrifugation. The protein, as well as the carbon and phosphorus content of the lipid, of several consecutive samples which had been subjected to as many as five washings was essentially the same as that of the preliminary unwashed stroma and the final dried product. It is evident, therefore, that the washing procedures used in this case did not remove measurable amounts of lipid from the stroma.

Only a few lipid analyses of stroma are available in the literature. Those prepared from human and sheep erythrocytes by adjustment of the pH with carbon dioxide demonstrated 22 and 17 per cent of alcohol-ether-extractable material, respectively (36). Human stroma prepared by a similar method (Thannhauser and Setz (37)) contained 26 per cent of substances extractable with 1:1 chloroform-methanol mixture. Appreciably higher values for lipid (48 per cent) have been noted in sheep stroma prepared by the ether hemolysis method of Chou (38). Recently, on the basis of weighed alcohol-ether extracts, as high as 71 per cent of lipid in dog stroma and 62 per cent in bovine stroma have been reported (22) on samples prepared by a combined procedure of acid precipitation at pH 5.5 and washing with a mixture of toluene and 0.01 N sodium hydroxide.

White and Monaghan (22) observed that it was necessary to employ prolonged alcohol-ether extractions of the stroma for lipid analysis. In the samples of sheep, bovine, and avian stromata discussed herein practically all of the lipid must have been extracted, for the total of the determined lipid, protein, and ash approximated 97 per cent of the initial weight (21). The total of these constituents accounted for only 89 and 85 per cent of the

<sup>4</sup> Fractionation and amino acid analyses of the posthemolytic residues as are now in progress in this laboratory may give definite information as to the composition and types of protein present.

human and horse stromata respectively, indicating the presence of appreciable amounts of undetermined constituents or additional lipids which are unusually resistant to alcohol-ether extraction.<sup>5</sup>

Observations indicated that characteristic protein to lipid ratios on the surface of the erythrocytes of different species may account for dissimilarities in physicochemical properties of the red blood cells (8, 10, 22). Related studies on erythrocytes and isolated stroma caused White and Monaghan (22) to suggest that erythrocytes having the highest electrophoretic mobility, sedimentation rate, and tendency for rouleau formation and the lowest isoelectric point, should have the lowest protein to lipid ratio on the surface. On the basis of the values for cataphoretic mobility (8), however, the protein to lipid ratios of stroma prepared from several types of mammalian erythrocytes do not substantiate the above suggestion. According to the above postulation bovine stroma should possess a high protein to lipid ratio, but our data demonstrate it to have the lowest ratio found for any of the stroma preparations.

From data on the permeability of inorganic and organic anions the influence of lipid areas seems to be more pronounced in the case of sheep and ox erythrocytes than those of man, rat, or mouse in which the factors of ionic size and hydrotropia seem to prevail (10). The data presented in Table II show a higher percentage of lipid in the stroma of the sheep and bovine erythrocytes than in the stroma of the human cells, results which are in accord with permeability studies.

From indications that protein films can be distended further without rupture than lipid films (10, 12), the critical volume of the erythrocytes (defined by Ponder (5) as "a maximum volume which is compatible with the integrity of the cell") may be related to the proportions of protein and lipid in the membrane. The higher protein to lipid ratio found in human stroma is consistent

<sup>5</sup> Samples of these same stroma preparations which were subjected to a prolonged alcohol-ether Soxhlet extraction gave lipid values comparable to those on alcohol-ether extracts prepared by standing at room temperature (with the exception of stroma Sample 32 in which 50 per cent more lipid was determined in the Soxhlet-extracted sample).



with the fact that human erythrocytes are more resistant to hypotonic changes than sheep and ox cells (4).

In spite of the characteristic differences in the absolute lipid values of the stroma preparations, the percentage distribution of the total lipid is consistent for all but avian stroma. Table II shows the distribution of lipids to be approximately 60 per cent phospholipid, 30 per cent free cholesterol, and 10 per cent neutral fat and cholesterol esters. The lipid pattern of the residues is essentially the same as that of the intact erythrocytes (Table III) except for the occurrence of slightly lower percentages of lipids in the form of cholesterol esters and neutral fat. In avian erythrocytes even more (75 per cent) of the lipids are phospholipids.

From the evidence at hand (18) the properties of bimolecular film coacervates, consisting of a mixture of phospholipids, simulate those of the erythrocyte membrane. De Jong and Bonner (18) have postulated that the bimolecular films of lecithin and sphingomyelin would be electrically stable in the physiological pH range but would not possess selective cation permeability and that the addition of cephalin would (in providing a surplus of negatively ionized groups) impart such properties.

Little is known of the biochemical rôle of the individual phospholipids, lecithin, cephalin, and sphingomyelin, owing to analytical difficulties of quantitatively differentiating them. The choline determinations, on hydrolyzed phospholipid extracts of erythrocytes and stroma (32), have furnished a more accurate means of calculating the distribution of phospholipids. Approximately half of the stroma phospholipids consists of lecithin and sphingomyelin; the remaining 50 per cent are in the form of cephalin, as shown in Tables I and II. Similar phospholipids are found in the stroma and in the erythrocytes of all but one of the different species studied (Table III). In avian red blood cells and stroma about 30 per cent of the phospholipid is present as cephalin. These results conform with those of other workers who have detected appreciable amounts of cephalin in the erythrocytes and stroma (36). There are indications that the remaining phospholipids are comprised chiefly of sphingomyelin with only small amounts of lecithin (36, 37). It appears from these indications that cephalin and sphingomyelin may serve as structural phospholipids, whereas lecithin may function metabolically.

#### SUMMARY

Detailed lipid studies including total fat, free and ester cholesterol, neutral fat, and phospholipids have been made on post-hemolytic residue (stroma) preparations of human, bovine, sheep, equine, and avian erythrocytes. These studies were accompanied by similar lipid and hematological observations of the intact erythrocytes.

Preliminary studies have indicated that practically all of the lipids of the red blood cells are separated with the stroma and are bound firmly enough to withstand removal by vigorous washing procedures.

There were marked hematological differences in the erythrocytes of the species studied, particularly in corpuscular measurements. Calculation of the lipid content representative of a single red blood cell indicated that the amount of lipid is closely related to the characteristic size of the cell: the large avian erythrocyte possessed a high lipid content; the small sheep cell much less lipid; while human and bovine red blood cells were intermediate in size and lipid composition.

The lipid composition of the dried posthemolytic residues—prepared by hemolysis and washing with sodium citrate buffer solution at pH 5.5—was similar for sheep, bovine, and equine erythrocytes, with 20 to 25 per cent of lipid. Stroma preparations of human red blood cells contained 10 to 15 per cent of lipid, while preparations of avian erythrocytes only contained an average of 3 per cent.

In spite of the differences in absolute values, the type of lipid mixture was essentially the same for all stroma samples and erythrocytes except that for avian blood. The total lipid of both stroma and erythrocytes was divided as 60 per cent phospholipid, 30 per cent free cholesterol, and the remaining 10 per cent as cholesterol esters and neutral fat. Approximately 50 per cent of the phospholipid consisted of cephalin.

The results have been discussed in relation to the physico-chemical properties and structure of the erythrocyte.

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# EFFECT OF DIBENZANTHRACENE ON VITAMIN A AND TOTAL LIPID OF MITOCHONDRIA

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In recent years more attention has been given to the extra-chromosomal factors in cell metabolism and reproduction. Moore (1), for example, working on the cleavage rate of egg cells, concluded that the cytoplasm of the ovum determines the speed of splitting and not the nuclear elements, nor the sperm which is all nucleus. MacDowell and Richter (2) and Murray and Little (3) have pointed to the importance of extrachromosomal factors in relation to the incidence of mouse leucemia and mammary tumors. Among the formed elements of the cytoplasm mitochondria have long been studied, but in many instances the conclusions drawn from the stained preparations alone have been too far reaching. Bensley and Hoerr (4) have shown that the statement commonly made in texts that lecithin is a constituent of mitochondria is erroneous. With suitable methods Joyet-Lavergne (5) has demonstrated the presence of vitamin A in these structures of the normal cells which he observed. To study the effect of a carcinogenic compound such as dibenzanthracene on the composition of mitochondria would require a method which permits closer analysis than that obtainable by staining.

## EXPERIMENTAL

The method of isolating the mitochondria was that of Bensley and Hoerr (4). Their procedure is to centrifuge the finely divided tissue suspension in the beginning at low speed and for short periods of time. This will precipitate the larger fragments as well as many broken and unbroken cells. It is unavoidable that some of the mitochondria are lost at this stage. When the larger

## 530 Dibenanthracene Effect on Mitochondria

particles have been thrown down by repeated centrifuging and discarded, the remaining suspension is centrifuged at higher speeds and for a greater length of time. The precipitates may be examined microscopically at any point in the procedure to determine their character and make-up. This aids in developing a routine suitable for any particular tissue.

In the case of liver tissue it will be found that such repeated centrifuging results in producing a supernatant liquid containing chiefly glycogen and protein in solution. From this the mitochondria are obtained by high speed centrifuging for a long time. The final precipitate is washed with saline until the washings give a negative test for protein. Finally, the mitochondria are washed with distilled water, dried as quickly as possible, and weighed.

In order to estimate the vitamin A content the dried mitochondria were repeatedly extracted with chloroform. The amount of vitamin in the combined extracts was determined by the method of Rosenthal and Erdélyi (6), also used by Lasch (7), and as modified by Andersen and Levine (8).

The total lipid was determined by the method used by Bensley and Hoerr (4). The dried mitochondria were extracted in a Soxhlet apparatus with hot absolute alcohol. This extract was evaporated to dryness *in vacuo* and the residue in turn extracted again with chloroform. The dried mitochondrial residue after extraction with alcohol was also extracted with hot chloroform. The two chloroform extracts were combined, evaporated to dryness, and the fatty substances weighed. Substitution of ether for chloroform in the above gave similar results for lipid material. Bensley and Hoerr (4), however, combined the alcohol and chloroform extracts, evaporated both together, and weighed the residues. This gives a slightly larger percentage of extracted material which is possibly due to non-lipid substances chiefly extracted with alcohol. All extractions were continued for 30 to 35 hours. All the lipids are removed by this method, indicating that mitochondria contain no lipid so intimately combined with other constituents as to prevent immediate extraction. To prove this the following method was adopted. After prolonged extraction the residues which remained after such treatment were digested in a solution of pepsin in dilute hydrochloric acid. After 36 to 48 hours incubation at 37° the solution was centrifuged at

high speed to precipitate any undigested material. This was washed and centrifuged at high speed a number of times to free it of any adhering pepsin and hydrochloric acid. The undigested residue was found to be less than 1 per cent of total mitochondria and furthermore was entirely insoluble in fat solvents after prolonged extraction.

In addition to lipids mitochondria contain a protein (4). It was found to give the usual protein color tests and a positive reaction for sulfur.

To ascertain the effect of 1,2,5,6-dibenzanthracene on hepatic mitochondria a colloidal solution of this hydrocarbon was injected intraperitoneally into rabbits. The colloidal solution was prepared by adding an acetone solution of the compound to an aqueous 0.5 per cent gelatin solution with subsequent heating to expel the acetone. Sodium chloride was then added to render it isotonic. The solution was made up to contain 3 mg. of the hydrocarbon in 10 cc. of final product, the usual amount given at each injection. When the number of injections and the elapsed time appeared suitable, the animal was killed, the liver removed, and the mitochondria isolated and analyzed. A control series of animals received injections of 0.5 per cent gelatin solution alone. This was prepared exactly as that in the preceding description except for the omission of dibenzanthracene. A third group received no injections whatsoever but were kept on the same diet and under the same conditions as all the others. Since a diet adequate in all respects is necessary for maintenance of health and gain in weight, this third group served the purpose of diet control as well as for the determination of normal values for mitochondria. All animals were fed Purina Rabbit Chow with occasional addition of vegetables and were weighed at frequent intervals. The rabbits selected for use in these experiments had an initial weight of 1000 to 1500 gm.

### *Results*

The following observations determined the location of vitamin A as being in the mitochondria. The various precipitates obtained in the successive centrifugings necessary for the separation of these structures were rapidly dried, extracted with chloroform, and the vitamin A content determined. It was found to be small

## 532 Dibenanthracene Effect on Mitochondria

indeed in the precipitates obtained at low speed and early in the procedure. However, later in the process of separation the precipitates showed on microscopic examination increasingly large numbers of mitochondria and on chemical analysis a simultaneous increase in vitamin A. The latter reached its maximum value in the final precipitate. The vitamin could also be detected by staining with antimony trichloride and microscopic examination.

It was found that in normal untreated rabbits on the above diet the lipid content of mitochondria ranged from 27.0 to 32.4 per cent. The values for vitamin A content in these varied from 249 to 910 U.S.P. units per 100 mg. of mitochondrial lipid. The animals marked N in Table I show some of the results obtained with the normal group. In the case of the control series (designated by C in Table I) the mitochondria contained comparable amounts of lipid and vitamin. Apparently the gelatin and the method of its injection or preparation had no effect. Both normal and control animals regularly gained weight.

In the group of animals receiving dibenanthracene a change in these values became evident. In the first place the percentage of lipid was in practically each instance above normal (animals marked D in Table I). In one experiment it rose above 52 per cent. More than this, the vitamin A content was especially reduced. In these rabbits the total amount of dibenanthracene given varied from 27 to 63 mg. administered over a period of 5 weeks. In from 1 to 14 days after the last injection analyses of mitochondria showed vitamin A to be entirely absent. Lastly, all of the animals lost weight rapidly and the number of deaths reached 60 per cent before any analysis could be made. There were no deaths in the normal or control series.

The changes in mitochondrial lipid and vitamin A could not be ascribed in the treated series to a possible decreased food intake. This was shown by results with animals on a starvation diet. These lost as much weight or even more in the same period of time as those receiving dibenanthracene, yet the mitochondrial lipid was normal and the vitamin A values were in the upper range for normal rabbits. Examples are marked L in Table I. It is apparent from this and other work (9) that the liver, although it may lose any excess stores of vitamin A, holds tenaciously for some time to a reserve when access is suddenly withdrawn.

TABLE I  
*Effect of Dibenzanthracene on Mitochondria*

Animal No.	Total dose of dibenzanthracene	Days after last injection	Mitochondrial lipid	Vitamin A per 100 mg. mitochon- drial lipid*
	mg.		per cent	units
N12	0	0	32.1	415
N2	0	0	30.7	290
N5	0	0	31.1	376
N9	0	0	31.3	318
N1	0	0	32.4	249
N4	0	0	28.9	262
N6	0	0	28.4	300
N7	0	0	29.8	260
N3	0	0	27.0	310
C8	0	1	30.4	345
C2	0	1	32.2	260
C7	0	7	31.6	910
C3	0	1	30.1	384
C10	0	5	29.2	430
C14	0	5	28.5	375
L34	0	0	32.4	327
L32	0	0	27.9	587
L36	0	0	30.1	415
L37	0	0	29.5	520
D6	48	4	34.2	0
D7	48	4	38.3	0
D15	54	1	40.1	0
D10	54	1	41.6	0
D16	63	13	36.5	0
D13	54	13	32.1	0
D14	63	14	52.3	0
D2	27	14	34.3	0
E27	9	1	33.1	13
E28	9	6	28.4	22
E18	9	9	40.1	0
E29	9	14	37.3	39
E20	9	23	45.0	42
E17	9	32	32.1	21
E26	9	40	27.3	206
E19	9	59	32.3	356

Animals marked N represent the normal untreated group; C, controls which received gelatin injections alone; L, received no food except water; D, dibenzanthracene-treated rabbits; and E, received dibenzanthracene for 1 week only.

\* As in Lasch's work (7) a biologically standardized preparation of halibut liver oil, containing 32,000 U.S.P. units per gm., was used in various dilutions as a standard.



## 534 Dibenzanthracene Effect on Mitochondria

In order to study the duration of the effects produced another series of animals was injected with a total of only 9 mg. of the compound, administered in three 3 mg. doses in 1 week. At various intervals of from 1 day to 9 weeks after the last injection the animals were tested. The lipid content of hepatic mitochondria rose to a maximum in about 3 weeks after the last injection, then fell to normal during the 5th week, and remained so thereafter. The vitamin A content was low as early as 1 day after the last injection and remained low up to the 5th week. Rising perceptibly in the 6th, it reached normal soon after in the following weeks. Typical results are illustrated by animals marked E in Table I. Loss of weight early in the series was also observed. Although rabbits vary as to their ability to withstand the toxic effects of dibenzanthracene, the above is an example of the recovery process. It appears from these latter experiments that the liver can regain in time the power to store vitamin A, if the injections of hydrocarbon be discontinued. This holds for the small dose used here. Occasionally an animal showed marked ability to withstand the effects of dibenzanthracene, but this was exceptional. It was also found that in normal rabbits on an ordinary diet, as given in the control series, the relation of liver to total body weight ranges from 2.2 to 3.4 per cent. This agrees with Polson's findings (10). In the dibenzanthracene-treated animals the liver was enlarged and this relation was well above 3.4 per cent and rose as high as 8.0. In addition to hepatic enlargement, which in itself may not always be of a distinctly pathological nature, the livers presented evidences of marked fatty changes. Further, it was noticed that if animals in poor physical condition and development were purposely selected for mitochondrial analysis, the hepatic vitamin A was below the normal values cited in Table I. This type of animal was peculiarly susceptible to injections of dibenzanthracene, dying after as little as 6 mg. of the hydrocarbon had been given. The same kind of animal when placed on an adequate diet for merely 1 week to 10 days rapidly gained weight and showed more normal values. After such treatment these animals would bear the injections very much better. Of course, this could not be ascribed to the vitamin alone.

It is interesting to note here Lasch's study (7) of the chemical

pathology of phosphorus poisoning. Although he did not carry out analyses of hepatic mitochondria, it was found that in spite of the noticeable fatty changes in the liver accompanying this condition, the vitamin A content of this organ was not diminished below normal. He was also able to increase the hepatic vitamin A by parenteral injection in animals poisoned with phosphorus. This is in sharp contrast with the results obtained with dibenzanthracene.

For the purpose of further comparison it was decided to study the effect of another cyclic compound. Analyses of hepatic mitochondria were then made on animals receiving intraperitoneal injections of *p*-aminoazobenzene. This organic compound has only two carbon rings and so far has been found to be non-carcinogenic for the animals upon which it has been tried (11, 12). It was found that repeated injections brought about no deaths. In fact they could be given five times weekly, whereas with dibenzanthracene the number of injections for the animals of the size used could not safely exceed three per week for any length of time lest all of them die before analysis. With *p*-aminoazobenzene all animals gained weight and some vitamin A could generally be found in the liver. The mitochondrial lipid was generally not above normal. It seems that the hepatic cells were not sufficiently injured to prevent the formation and storage of the vitamin.

Another group of rabbits was given three 3 mg. injections of *p*-aminoazobenzene in 1 week. No further injections were made. At intervals from 1 day to 3 weeks the hepatic mitochondria were analyzed. Although the livers were enlarged, the lipid content was normal in most instances and there was only a moderate reduction in vitamin A soon after the last injection. Recovery to normal values of vitamin took place in 1 week.

After obtaining these results it appeared conceivable that certain initial steps in the metabolism of the pentacyclic hydrocarbon, dibenzanthracene, might be linked to vitamin A or at least its precursor carotene. This led to a number of experiments in which rabbits, receiving repeated injections of dibenzanthracene at intervals which the previous work had shown would lead to absence of vitamin A in hepatic mitochondria, were injected parenterally with the vitamin. A subcutaneous injection of 30,000 U.S.P. units (in the form of halibut liver oil) was given at the same

## 536 Dibenzanthracene Effect on Mitochondria

time as each intraperitoneal one of 3 mg. of dibenzanthracene. At times one or more additional injections of vitamin were made. The results showed no deaths in the entire series; all except two gained weight; the mitochondrial lipid was somewhat closer to normal; but the vitamin A values still remained low. Apparently the animals were able to utilize generally the vitamin given parenterally, as shown by the results just mentioned. The liver, however, was not capable of storing much vitamin A in the presence of dibenzanthracene.

TABLE II

*Effect of Dibenzanthracene Plus Carotene or Vitamin A on Mitochondria*

Animal No.	Total dose of dibenzanthracene	Total dose of carotene or vitamin A	Mitochondrial lipid	Vitamin A per 100 mg. mitochondria lipid
	mg.	1000 units	per cent	units
T55	6	60	35.4	0
T53	15	150	39.2	0
T58	21	360	28.9	154
T57	21	360	32.7	0
T56	24	240	31.5	17
T51	27	450	34.6	10
T63	30	480	35.7	0
T47	30	480	31.0	16
V62	6	85	32.8	0
V63	12	175	32.7	379
V65	21	265	33.2	48
V64	24	390	29.5	30
V61	27	450	34.7	15
V48	27	450	32.8	18
V46	30	480	34.1	26
V60	30	480	30.5	0

Animals marked T represent carotene-treated group; those marked V, the vitamin A-treated group. Both groups received dibenzanthracene.

It might be argued that the absence of vitamin A in the liver of an animal receiving dibenzanthracene and on an ordinary diet is explained by the inability of this organ during such treatment to split carotene obtained from the diet into vitamin A. Therefore, it was decided to inject another group of animals with carotene and the usual 3 mg. of dibenzanthracene. Other work (13) has indicated that parenteral injections of carotene are utilized by animals to some extent when placed on a simple

vitamin A-free diet. Improvement in general physical condition and amelioration of certain avitaminosis took place. This was confirmed by Greaves and Schmidt (14). The results in this series of tests showed 40 per cent of deaths before analysis could be made; there was a loss of weight in all instances except two; the mitochondrial lipid remained fairly normal; but the hepatic vitamin was low. It seems that the liver was not able readily to form sufficient vitamin from the carotene administered parenterally under these conditions.

The results in the two last sets of experiments are illustrated in Table II in which the series marked T represents the carotene-treated and that marked V is the vitamin A-treated group.

Further work on the effect of carcinogenic compounds is in progress.

#### SUMMARY

1. The vitamin A of hepatic mitochondria was estimated.
2. Repeated intraperitoneal injections of 1,2,5,6-dibenzanthracene markedly reduced the vitamin A and increased the total lipid of hepatic mitochondria. Enlargement of the liver and loss of weight accompanied these changes.
3. The liver regained the power to store vitamin A after some time when the dose of the hydrocarbon was small and provided it was discontinued.
4. In all respects mentioned above *p*-aminoazobenzene had much less effect than dibenzanthracene.
5. The liver was apparently not able to split carotene administered parenterally while the animal was receiving dibenzanthracene. Under the same conditions parenteral injections of vitamin A brought about little increase of vitamin in the hepatic mitochondria, although certain generally beneficial results were observed.

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## THE DETERMINATION OF SULFANILAMIDE (*p*-AMINO-BENZENESULFONAMIDE) IN BIOLOGICAL MEDIA

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Studies of possible methods for the determination of sulfanilamide concentrations in various media were begun some time ago. The oxidation method suggested (1) is specific, giving rise to *p,p'*-disulfamidoazobenzene. This method is satisfactory only at or above concentrations of 50 mg. per cent. Schiff base formation (2) gives rise to specific, yellow-colored substances but these reactions are not applicable to dilute solutions. The phenol reagent of Folin and Ciocalteu (3) is non-specific. Sulfanilamide solutions may be titrated with mercuric nitrate as in the Liebig method for the determination of urea (4); in the presence of excess sulfanilamide, acidic solutions of mercuric nitrate precipitate a white complex<sup>1</sup> (involving sulfanilamide) upon the addition of sodium carbonate.<sup>2</sup> This method is cumbersome and non-specific. The indophenol reaction with a saturated bleaching powder solution is neither specific nor sensitive, giving transient colors above 25 mg. per cent. These reactions may be used for a qualitative identification of sulfanilamide.

A rather specific color test with good, although not excellent, sensitivity is obtained by boiling sulfanilamide solutions with sodium hypochlorite in excess alkali.<sup>3</sup> At high concentrations

<sup>1</sup> It has been pointed out (5) that sulfanilamide is a variant vinylog of urea, giving a similar xanthidrol reaction. It has also been found to give a formol condensation.

<sup>2</sup> In examining extraction residues toxicologically, note that sulfanilamide gives a precipitate with Millon's reagent.

<sup>3</sup> The reagent was prepared by shaking 4.0 gm. of bleaching powder (20  $\pm$  per cent available chlorine) from time to time in 100 cc. of water. After 3 hours, 10 per cent sodium hydroxide was added in excess of maxi-

(25 mg. per cent) sulfanilamide solutions give a red color, a golden yellow at low concentrations (5 to 10 mg. per cent), and a canary-yellow at the lower limits of sensitivity (1 to 3 mg. per cent). Tyrosine gives a trace of color at 20 mg. per cent, although amino acids and phenols do not. Aniline gives the usual aniline black test with this reagent. Sulfanilic acid gives the same color reactions given by sulfanilamide. Thus, the reagent distinguishes between certain arylamines, but will not differentiate para-substituted aromatic amines. It is a good confirmation of diazotization methods for toxicological procedures.

Since these methods lacked sensitivity, diazotization procedures were studied.  $\beta$ -Naphthol, first used by Fuller (6) as a  $\beta$ -coupling component, was tried. The colors developed were stable, soluble in alkaline solution, and nitrosation was avoided by coupling in the presence of alkali. However, the  $\beta$  component did not appear to be sufficiently chromogenic. Marshall, Emerson, and Cutting (7) then reported an acid-coupling method with  $\alpha$ -dimethylnaphthylamine as the  $\beta$  component. This method involved an alcoholic precipitation of the blood proteins. We have had difficulty in matching the colors obtained in blood and urine specimens with the color standard by this method. This may be due in part to a difficulty in obtaining satisfactory shipments of  $\alpha$ -dimethylnaphthylamine.<sup>4</sup> However, in the presence of sulfanilamide, alcoholic blood filtrates from icteric patients slowly developed a diluted van den Bergh reaction (8) when diazotized. It is significant that cases of hepatitis with jaundice have been reported as a result of prolonged sulfanilamide therapy (9). Since an excess of nitrous acid is used in this method, amounts of nitroso- $\alpha$ -dimethylnaphthylamine varying with the concentration of sulfanilamide, urea, etc., are formed. These, and the yellow color of the filtrates, due to carotinoids, bilirubin, etc., contribute to color admixture. More recently Marshall modified this method (10).

Chromotropic acid (Eastman) appears to be a satisfactory  $\beta$  component. It is water-soluble, giving a neutral solution at a

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mum precipitation and the reaction mixture was filtered. The test was performed by boiling 2 volumes of the clear filtrate with 1 volume of sulfanilamide solution for 1 minute.

<sup>4</sup> Personal communication from Dr. Marshall.

concentration of 50 mg. per cent. The solution is colorless and remains so for 2 weeks or more if kept stoppered in a dark bottle. Photochemical oxidation imparts a deep yellow color to the solution within 24 hours. In the presence of alkali this change is very rapid (1 to 2 hours) and the resulting color is a bluish red. This color simulates a positive test for sulfanilamide and appears to be due to a quinonoid substance which is tautomeric with that producing the yellow color, since the two are interchangeable, depending upon the pH. Consequently, neutral chromotropic acid solutions having a yellow tint should not be used. Further, since the  $\beta$  component is a naphthol, ferric ions and other oxidizing agents must be avoided.

The diazotization of sulfanilamide solutions at the dilutions found is complete within 3 minutes. If an excess of sulfanilamide (over 25 mg. per cent) is present in the unknown, the excess gives rise to tetrazo formation, characterized by a deep reddish blue color. This condition is thus easily detected and may be corrected by diluting the unknown prior to coupling.

Sodium hydroxide is unsatisfactory for coupling to chromotropic acid when used in excess. Colors developed in alkaline media drift, and this is due to oxidation of the chromotropic acid.<sup>5</sup> Sodium carbonate is used as the coupling alkali. Coupling is carried out with a minimum amount of  $\beta$  component, at a pH neutral to red and blue litmus. In this way nitroso formation and oxidation changes are avoided. The colors developed may be matched with excellent precision in a colorimeter and become only slightly yellow in tint upon standing overnight. This change is slight and does not alter the final reading more than the limit of error within 6 to 7 hours as determined by the preparation of fresh standards. If the tests are negative, they remain negative,

<sup>5</sup> A sulfanilamide solution was diazotized and coupled to chromotropic acid in the presence of excess alkali, and at the same time a similar procedure was carried out in the absence of sulfanilamide. The second reaction developed a bluish red color within 2 hours. After 2 hours the first reaction was repeated and this solution was combined with the second specimen. The combined second and third solutions compared quite well colorimetrically with the first specimen after appropriate dilution. It is interesting that similar changes did not occur in Folin-Wu filtrates, presumably owing to the reducing substances present.



becoming slightly yellow upon long standing. The test is sensitive to 1 part in 2 million.

Examination of over 300 Folin-Wu (11) filtrates from a wide variety of pathological blood specimens by this method has failed to reveal any interfering substance. However, the newer derivatives of sulfanilamide, 4-(4'-aminobenzenesulfamido)-benzenesulfonamide (12) and methylated analogues, give the same test. Further, any aminonitrophenol in blood or urine as a result of thermol (dinitrophenol) administration (13) will give a positive reaction.

In thirty-six control experiments, sulfanilamide solution (100 mg. per cent) was added to pooled whole blood (oxalated) such that it was present in concentrations of 10 and 20 mg. per cent. Individual precipitations of the blood proteins were carried out by the Folin-Wu method (modified by combining the tungstate and water prior to laking the blood). The sulfanilamide was then determined as follows:

To 5 cc. of filtrate, 1 cc. of sodium nitrite (0.1 per cent) and 1 cc. of hydrochloric acid (0.1 N) were added. The solution was mixed and allowed to stand for 3 minutes. Then 1 cc. of sodium carbonate (1.0 per cent) was added (the reaction must be neutral to red and blue litmus), followed immediately by 1 cc. of chromotropic acid (50 mg. per cent). The reactants were mixed by inverting once and the colors were compared directly in a colorimeter *versus* the appropriate standard (either 1 or 2 mg. per cent). All readings were checked by two or more observers.

The recovery of sulfanilamide was quite constant. The data indicated an average recovery of 81 per cent ( $\pm 3$  per cent, maximum deviation 7 per cent) of the sulfanilamide added. The per cent unrecovered was the same at concentrations of 10 and 20 mg. per cent, and was independent of the time that the sulfanilamide remained in contact with the blood. The fraction unrecovered was coprecipitated with the serum proteins. This was demonstrated by alcoholic extraction of five precipitates. The evaporated alcoholic extract, diluted with an equal volume of water, diazotized and coupled to chromotropic acid accounted approximately for the unrecovered 19 mg. per cent. (These colors were matched by intensity, since the alcoholic filtrates were yellow in tint.)

Ten similar control experiments in which the Haden modification (14) of the Folin-Wu protein precipitation was used were performed and 76 per cent ( $\pm 1$  per cent, maximum deviation 3 per cent) of the sulfanilamide was recovered. The data showed excellent agreement among themselves and are those expected by comparison with the data from the Folin-Wu filtrates, since the Haden modification gives a more nearly neutral filtrate. Sulfanilamide is an arylamine whose solubility increases inversely with pH, within limits. Hence, the coprecipitation should increase as the filtrate approaches neutrality, since coprecipitation increases inversely with the solubility of the coprecipitated substance.

In twenty control experiments with trichloroacetic acid (1 volume of blood was added to 9 volumes of 5 per cent trichloroacetic acid) excellent consistency was observed. At concentrations of 10 mg. per cent, 98 per cent ( $\pm 1$  per cent, maximum deviation 2 per cent) of the sulfanilamide was recovered. At concentrations of 20 mg. per cent, 95 per cent ( $\pm 1$  per cent, maximum deviation 4 per cent) was recovered. This increase in the recovery of sulfanilamide as compared with the Folin-Wu and the Haden modification for the precipitation of the blood proteins is a function of the increased filtrate acidity and probably the increased particle size (15).

In the trichloroacetic acid procedure, the increased filtrate acidity required an increase in alkali to bring the coupling pH to neutrality. 5 cc. of the filtrate were titrated to neutrality with 0.1 N NaOH; the volume was adjusted to 10 cc. and this was diazotized, etc., as above. The colors were compared with 10 cc. of standard solutions at 1:20 dilution. The simpler procedure of adding 1 cc. of 9 per cent sodium carbonate in place of the 1 per cent solution used with Folin-Wu filtrates gave pH variations.

The wide applicability and the facility of manipulation of the Folin-Wu filtrate give that procedure certain advantages over the trichloroacetic acid method, even though the final value must be divided by the factor 0.81.

For urine determinations, the specimens must not be preserved with formalin, since sulfanilamide readily undergoes formol condensation in neutral or slightly alkaline solutions.<sup>6</sup> The deter-

<sup>6</sup> This reaction is being studied and will be reported in detail elsewhere.

mination of free sulfanilamide in urine is essentially that used in blood filtrates. The urine was diluted 1:100 or 1:200, so that the final concentrations were 1 to 2 mg. per cent sulfanilamide. To 5 cc. of the diluted urine, 1 cc. of  $\text{NaNO}_2$  and 1 cc. of hydrochloric acid were added. The reaction mixture was shaken and allowed to stand for 3 minutes (slightly acid to Congo red). Then 1 cc. of sodium carbonate and 1 cc. of chromotropic acid were added, and the tube was inverted once and the colors read directly in a colorimeter at a pH neutral to red and blue litmus.

In twenty determinations on normal urine at 1:50 dilution, containing known amounts of sulfanilamide, an average of 87 per cent of the sulfanilamide was recovered as determined by comparison with standards prepared in distilled water. The deficit is due to inexact color comparability. Urinary pigments contribute largely to this. Highly pigmented urines gave the lowest readings at equal dilutions of urine volume. The effect of the urinary pigments is largely if not wholly circumvented at dilutions of 1:100 or higher.

There is a substance or substances in urine which becomes yellow upon the addition of sodium carbonate prior to coupling. This substance is removed by treatment with permutit according to the method of Folin and Bell (16). At a concentration of 1 mg. per cent approximately half of the sulfanilamide was retained by the permutit, but the adsorption was negligible at 100 mg. per cent. This is a concentration frequently found in urine.

Assuming the interfering substance to be largely ammonia, the ammonia was aspirated out of control samples of urine by the method of Folin (17). Urinary pigments were not removed by this procedure, but excellent matches were obtained. In a series of ten such determinations 98 to 99 per cent of the sulfanilamide was recovered. Hence, ammonia is an interfering substance and this imparts yellow casts, to some extent, to the final color by diazoamino (18) formation and reduces the final concentration by equivalent amounts. For clinical purposes this may be neglected when urine samples can be diluted 1:100 or more; *i.e.*, if the concentration exceeds 40 mg. per cent.

Since this drug is largely eliminated as the acetyl derivative (7, 19), alkaline hydrolysis was studied to remove ammonia as well as to liberate the arylamine. Acetylsulfanilamide (20) was

dissolved in warm water to give a 100 mg. per cent solution. No hydrolysis occurred during the preparation of the solution, since it gave negative reactions with the test described herein. 1 cc. samples of the solution were warmed in boiling water for varying lengths of time with 1 cc. of N sodium hydroxide. The solutions were cooled by dilution, titrated to neutrality with 0.1 N hydrochloric acid, diluted to 80 cc. (equivalent to a 1 mg. per cent solution of sulfanilamide), and 5 cc. of these solutions were diazotized and coupled as described above. A smooth exponential curve was obtained and hydrolysis was complete after 50 minutes of heating. A similar specimen treated with 1 cc. of N hydrochloric acid gave complete hydrolysis within 30 minutes. Similar data were obtained with urine specimens containing known amounts of acetylsulfanilamide. Better color matches were obtained at low concentrations with alkaline hydrolysis.

Marshall, Cutting, and Emerson (19) reported a selective adsorption of acetylsulfanilamide by charcoal. From our experience with the coprecipitation of sulfanilamide it appeared probable that this same phenomenon would occur to an equal or greater extent with acetylsulfanilamide.

Acetylsulfanilamide (100 mg. per cent) was added to pooled whole blood (oxalated) to give 10 and 20 mg. per cent solutions. Five Folin-Wu precipitations were carried out at each concentration. 5 cc. of each filtrate were then hydrolyzed by warming for 30 minutes with 1 cc. of N hydrochloric acid. The filtrates were then neutralized with N sodium hydroxide, and the volumes adjusted to 10 cc., and these were then diazotized and coupled in the manner described above. At 10 mg. per cent of the acetylsulfanilamide in whole blood, only 48 per cent was recovered and at 20 mg. per cent, only 47 per cent was recovered.

With trichloroacetic acid as the protein precipitant slightly better recovery was obtained. In ten precipitations of whole blood containing 10 mg. per cent of acetylsulfanilamide, an average recovery of 64 per cent ( $\pm 1.5$  per cent, maximum deviation 3.5 per cent) was observed. The 1 cc. of N hydrochloric acid must be added for hydrolysis, since most of the trichloroacetic acid is decomposed by the 30 minutes of heating.

It is possible to determine free sulfanilamide concentrations in blood with a trichloroacetic acid precipitation. The filtrate may

be hydrolyzed and the increase in free sulfanilamide may then be divided by 0.64 to correct for coprecipitation. However, such a large correction factor does not seem admissable. For the present, the method is limited to the determination of free sulfanilamide in blood. Work is in progress with a view to a more satisfactory extraction of the acetylsulfanilamide.

During the course of this investigation it became possible to study two normal persons who had been given large single doses (60 grains) of sulfanilamide. Blood samples analyzed by this method showed maximum sulfanilamide concentrations of 7.1 and 8.3 mg. per cent. These were attained 4.5 and 1.5 hours, respectively, after administration of the drug. The concentrations dropped to 2.3 and 2.7 mg. per cent after 24 hours. The rate of excretion varied widely and approximately 60 per cent of the sulfanilamide was recovered. Urinary elimination was complete in 2 to 3 days.

This problem was suggested by Dr. Alexander O. Gettler of Bellevue and Allied Hospitals. Thanks are further due Dr. Gettler for his many suggestions and for reading the manuscript. Thanks are due to Dr. Solomon Weintraub, Pathologist, Dr. Louis T. Wright, Visiting Surgeon, and Dr. Jesse G. M. Bullowa, Visiting Physician of Harlem Hospital for their kind interest in this investigation.

#### SUMMARY

Various methods for the determination of sulfanilamide have been discussed. Sodium hypochlorite has been shown to differentiate sulfanilamide from certain arylamines. Adsorption of sulfanilamide by permutit has been observed. A diazotization procedure for the quantitative determination of sulfanilamide concentrations in blood and urine has been devised. The hydrolysis of acetylsulfanilamide has been studied and by this means its concentration in urine has been determined. Urinary pigments and ammonia have been shown to produce low values in urine determinations, and means of circumventing these interfering substances have been considered. An objection to the use of formalin as a urine preservative has been pointed out.

The coprecipitation of sulfanilamide and acetylsulfanilamide has been studied. This phenomenon has led to difficulties in the

determination of acetylsulfanilamide in blood. A tentative method for its determination has been considered but this phase of the problem is being studied further.

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# THE SOLUBILITY AND CHEMICAL AND PHYSICAL ABSORPTION OF NITROGEN GAS IN AZOTOBACTER CELLS

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Under suitable conditions the free living, aerobic soil organism *Azotobacter* fixes nitrogen at a surprisingly rapid rate, considering that the fixation occurs at ordinary temperatures and pressures. The maximum rate of fixation by *Azotobacter vinelandii* that has been obtained in liquid cultures at 36° and 0.78 atmosphere of nitrogen pressure corresponds to a fixation of about 0.04 gm. of nitrogen per gm. of cell dry weight per hour. The rate of fixation by inorganic catalysts is about 6 gm. per gm. of catalyst per hour in the Claude process ( $t = 500-650^{\circ}$ ,  $p = 900$  to 1000 atmospheres) and 0.3 to 0.4 gm. per gm. of catalyst per hour in the Haber process ( $t = 500-600^{\circ}$ ,  $p = 200$  atmospheres). The products of fixation by *Azotobacter* are in good part bound up in the cell in the form of protein-like material.

The kinetics of nitrogen fixation are specifically interpretable by the Michaelis-Menten theory of enzyme action (13, 11, 10) according to the following representation



where  $P$  does not necessarily represent cell material, the final product of nitrogen fixation, but possibly some intermediate product formed practically irreversibly. Letting  $v$  and  $V_{\max}$  represent respectively the observed and maximum velocities of the second reaction,  $P_{\text{N}_2}$  the pressure of nitrogen, and  $K_{\text{N}_2}$  ( $= 170$



mm. of Hg) the dissociation constant of the enzyme-substrate complex, we obtain

$$v = \frac{V_{\max} P_{N_2}}{K_{N_2} + P_{N_2}}$$

which is an accurate expression of the experimentally observed kinetics (11).

Between 20–30° the equilibrium reaction, Equation 1, has been shown to have a very low heat of reaction,  $\Delta H = 0$  ( $\pm 600$ ) calories ((2) Equations 8 and 13), and the irreversible reaction, Equation 2, a fairly large temperature coefficient ( $E_a = 19,300$  calories; (2) p. 38) which becomes even larger at temperatures below 17° (unpublished data). These facts make it possible for the irreversible reaction to be made practically negligible by the use of low temperatures, and, theoretically at least, to ascertain the amount of nitrogen combined with a cell enzyme component such as  $E$ , by performing a vacuum extraction on nitrogen-equilibrated cell suspensions.<sup>1</sup> Since the nitrogen combined in this manner should yield a hyperbolic isotherm comparable with that obtained kinetically (11), we could distinguish it from the nitrogen dissolved according to Henry's law by making sufficiently exact nitrogen gas determinations at several nitrogen pressures. For the cell concentrations employed the sensitivity of the method made it possible to detect the presence of an amount of chemical adsorption equivalent to 0.01 of the calculated amount of nitrogen required to form a monomolecular layer on the surfaces of the cells, if the nitrogen uptake agreed quantitatively with the kinetic isotherm. Positive results in such an experiment would be highly important generally, from the standpoint of the mechanism of enzyme action, and specifically

<sup>1</sup> The maximum error that might arise due to fixation decreasing the amount of dissolved or reversibly combined nitrogen gas during the interval between equilibration and analysis has been estimated on the following basis. For a velocity constant of fixation of  $10^{-4}$  (1/1000 that at ordinary temperatures) about  $3 \times 10^6$  molecules of nitrogen would be transformed per hour per cell. While this figure is of the order of the sensitivity involved in the analytical procedure, it is to be noted that analyses were always carried out within an hour after equilibration. Only random variations occurred on duplicate analyses carried out 20 minutes apart.

because they would give the proposed mechanism of nitrogen fixation a much more definite quantitative physical significance. Negative results, on the other hand, would show that the isotherm was not due to an extensive surface adsorption, and would establish an upper limit to the number of independently acting enzyme (nitrogenase) units in an *Azotobacter* cell.

In the present experiments isotherms for the solubility of nitrogen in water, nutrient medium, medium + sucrose, and aqueous suspensions of *Azotobacter*, legume nodule bacteria, and yeast were determined over the range 0.7–25°. In addition uptake of nitrogen and argon at 0° and –183° by dried cells was determined.

### *Apparatus*

A thermostat holding to  $\pm 0.03^\circ$  at temperatures as low as 0° contained rotating double tonometers for saturation of the liquids with gases. Fig. 1 shows the modified Van Slyke-Neill extraction chamber used with the Van Slyke blood gas apparatus (14). This chamber accomodates large samples of liquid that may be recovered without contamination with reagents and permits analyses of dissolved gases at temperatures considerably below room temperature (*cf.* Shepherd (16)). Water from the thermostat was pumped through the jacket when low temperature analyses were performed. The sample and reagent pipettes were made according to Van Slyke and Neill (18).

The uptake of nitrogen by dried cells was kindly carried out by Dr. Brunauer of this laboratory, working with Dr. Emmett, and with use of their low pressure adsorption apparatus (4).

### *Materials and Reagents*

1.5 *N* potassium hydroxide was made gas-free by repeated extraction *in vacuo* and transferred, without even momentary contact with air, to a storage pipette. 0.8 cc. of this solution was used for CO<sub>2</sub> absorption.

Gas-free hyposulfite solution was prepared according to Van Slyke ((17) p. 575). 0.8 cc. of this solution was used for O<sub>2</sub> absorption.

*Gas Mixtures*—The four gas mixtures used were 18.03 per cent nitrogen (inert gases) in oxygen, 47.90 per cent nitrogen in oxygen, air, and nitrogen containing <0.1 per cent O<sub>2</sub> and <0.1 per cent

argon. These standard mixtures were compressed in suitable containers.

In Tables I to IV it will be obvious which mixture was used, since the total pressure was atmospheric and therefore the partial pressures of  $N_2$  were approximately 135, 360, 590, and 750 mm. respectively.

*Azotobacter*, *Rhizobium*, and *Yeast Cells*—The physical properties of the cells employed in these experiments have been described (9). The physiological properties follow. No observations on

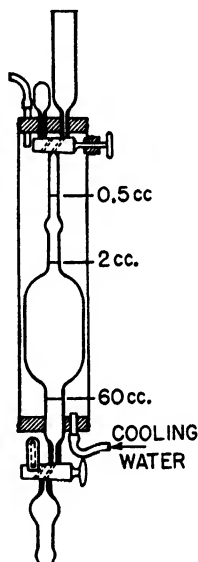


FIG. 1. Modified Van Slyke-Neill extraction chamber

the metabolic activity of the yeast and *Rhizobium* cells used in the  $N_2$  uptake experiments were made, since their use was intended only to afford controls in regard to the uptake of nitrogen by ordinary non-fixing, one-celled plants. *Rhizobia* are active in nitrogen fixation only when growing symbiotically with their host plants.

However, in order to evaluate the results of the  $N_2$  uptake experiments in relation to the proposed nitrogen fixation mechanism in *Azotobacter*, it was desirable to know to what extent the actual experimental *Azotobacter* material employed was capable of nitro-

gen fixation. The nitrogen fixation or growth activity of the *Azotobacter* was therefore determined, both turbidimetrically and by respiration rate increase, on diluted material. These experiments of 6 to 8 hours duration were performed on the Warburg apparatus at 31° in both free and fixed nitrogen, and at several cell concentrations (the usual values of  $g$ , the specific rate of increase in respiration rate, turbidity, etc., range from 0.15 to 0.25 for free nitrogen and 0.20 to 0.35 for fixed nitrogen; cf. (2, 11)).

*Experiment 38*—This sample showed only a low nitrogen-fixing ability. The value of  $g$  in fixed nitrogen, measured by both respiration rate and turbidity increase, was more than one-half the usual value, but in free nitrogen the  $g$  was of the order of one-tenth the usual value. This observation perhaps implies that these cells contained a low concentration of nitrogenase, the enzyme presumed to combine reversibly with  $N_2$  gas.

*Experiment 46*—Comparison of the increase in respiration rate and turbidity showed that the two methods did not parallel each other. The  $g$  values determined turbidimetrically were, of course, considered the better basis for estimating the fixation activity. Values of  $g$ , so obtained, were one-fourth to one-half the usual value in either free or fixed nitrogen.

*Experiment 60*—Qualitatively an increase in turbidity was obtained in both free and fixed nitrogen. The order of activity, however, appeared to be more nearly like that of Experiment 38, so that here again the amount of nitrogenase per cell might possibly be expected to be low. The c.mm. of  $O_2$  consumed per mg. of dry weight per hour was measured for this culture at both 31° and 0.7° and found, respectively, to be 600 and 1.1, each accurate to about 10 per cent. If the rate of respiration were considered to treble for each temperature increase of 10°, the value 30 would be expected at 31° instead of 600. It is therefore evident that there occurs considerable deviation from the situation existing between 20–30°, where a fairly constant temperature increment of 19,330 calories was obtained (12).

*Experiment 64*—Respiration rate and turbidity observations were made with both diluted, centrifuged cells and uncentrifuged cells. Typical respiration rate  $g$  values were observed for both materials whether free or fixed nitrogen was used. The turbidity  $g$  values were, however, about one-half the respiration rate  $g$

values for the centrifuged cells, but only about 20 per cent less for the uncentrifuged cells. It appears that although a fairly satisfactory amount of nitrogenase per cell was assured by the rate studies, the supercentrifugation and resuspension apparently did injure the cells to a certain extent, in some general manner.

As previously mentioned, numerous factors affect the activity of *Azotobacter* and it is not surprising that cultures obtained by the 17 liter culture technique should yield values of  $g$  different from those obtained when the usual 100 cc. culture method is used (2). The most probable explanation for the fact that the respiration and turbidity  $g$  values do not agree is that the method of culture produces a mixed population, consisting of cells respiring only, cells respiring and reproducing normally, and cells respiring and reproducing subnormally. When placed under the conditions employed in the Warburg technique, the distribution of the population no doubt gradually changes subject to the new conditions. These observations of low rates of fixation, which, of course, fail to supply evidence that the amount of nitrogenase per cell was as high as that usually encountered in the kinetic studies, do not necessarily *prove* that the nitrogenase content of the cells was low. This readily follows when one considers that the rate of growth may be low due to any one of a large number of steps in the growth process being affected. In fact, the fixation step has been shown, generally, to be relatively less sensitive to factors such as culture condition, inhibitors, etc., than the respiration and growth processes (2). Hence, it is not proper to conclude that cells temporarily reproducing only slowly or not at all are deficient in nitrogenase. This position is possibly further supported by the reported stability of nitrogen-fixing cell-free extracts of *Azotobacter* (Bach and coworkers (1)). Only in Experiment 38 where a considerable difference between the free and fixed nitrogen  $g$  values was found does the conclusion that the cells were low in nitrogenase seem at all warranted.

The view that the *Azotobacter* cells obtained by the 17 liter culture technique contained 50 to 100 per cent of the amount of nitrogenase encountered in the previous kinetic studies will be adopted, although a certain amount of caution is admittedly necessary in drawing far reaching conclusions.

*Saturation of Liquids with Gas Mixtures*

The experimental liquid, generally about a 40 cc. sample, was distributed between the 50 and 500 cc. tonometers in proportionate amounts. The double tonometer was then evacuated to about 25 mm. of Hg, filled with a definite gas mixture, rotated for 10 to 20 minutes to remove traces of air from the liquid, reevacuated, and refilled with the gas mixture. Two evacuations and refillings sufficed to prevent variations in the composition of the gas phase that would affect the results outside the error of the analytical solubility measurements. Alteration of nitrogen pressure for any of the gas mixtures due to different solubilities of nitrogen and oxygen was less than 0.5 mm. of Hg. The pressure in the tonometers was brought to atmospheric by opening the stop-cock at intervals and allowing the pressure to fall to atmospheric. The tonometers were rotated at the rate of about 15 R.P.M., for not less than 3 hours. This time was adequate for the attainment of equilibrium.

*Analysis of Liquids*

The general features of the procedures for determination of dissolved gases described by Van Slyke and coworkers (14, 17, 18) were employed. The pipettes used to transfer the liquid to be analyzed from the equilibration tonometers to the extraction chamber held 14.37 cc. at 25°. The small accessory chamber (Fig. 1) was used to store extracted gas while subsequent extractions were being made. After complete extraction thus attainable the liquid could be recovered and the chamber even rinsed before measurement of the gas. Reagents were generally introduced through the bottom stop-cock. The analysis temperature was within 5° of the saturation temperature in all cases.

*Calculations*

The  $N_2$  content of the liquid calculated as volume per cent of nitrogen was obtained by multiplying the nitrogen pressure by

$$\frac{100 a (A + b)}{AS 760(1 + 0.00384t)}$$

where the volume to which the gas was brought for measurement  $a = 2.811$  or  $0.446$  cc., the volume of the extraction chamber

$A = 55.7$  cc., the volume of the storage chamber  $b = 1.3$  cc.; and the volume of the sample  $S = 14.37$  cc. All pipette and chamber volumes were determined at  $25^\circ$ ; however, since the gas analyses were always made at a temperature near that at which the sample was transferred, the same volumes could be used to calculate the factor for all temperatures.

The following symbols and definitions will be used throughout this paper.

$\alpha$  = cc. of  $N_2$  ( $0^\circ$ , 760 mm.) dissolved per cc. of liquid under 760 mm. of  $N_2$  pressure.

$\alpha^\circ$  = cc. of  $N_2$  ( $0^\circ$ , 760 mm.) dissolved per gm. of water in the liquid under 760 mm. of  $N_2$  pressure. The values of  $\alpha^\circ$  used in the calculations indicated below were chosen from Tables I and III according to the temperature and suspending medium employed (see also foot-note ||, Table IV).

$\alpha_{\text{wet cells}}$  = cc. of  $N_2$  ( $0^\circ$ , 760 mm.) taken up by 1 cc. of wet cells under 760 mm. of  $N_2$  pressure.

$\alpha_{\text{dry cells}}$  = cc. of  $N_2$  ( $0^\circ$ , 760 mm.) taken up by 1 gm. of suspended cells (dry weight) under 760 mm. of  $N_2$  pressure.

The solubility coefficients,  $\alpha$  and  $\alpha^\circ$ , were calculated by the equations,

$$\alpha = \frac{760 [N_2]}{100 p}$$

$$\alpha^\circ = \alpha C_{H_2O}$$

where  $[N_2]$  represents the volume per cent of  $N_2$  found in the liquid,  $p$  the pressure of nitrogen in mm. of mercury, and  $C_{H_2O}$  the gm. of water per cc. of liquid (Table I (9)).

$[N_2]_{\text{subscript}}$  = nitrogen gas content for the material denoted by the subscript either calculated from the  $C_{H_2O}$  and the appropriate  $\alpha^\circ$  or actually measured. The units are cc. of  $N_2$  per cc. of liquid material or cc. of  $N_2$  per gm. of dry material unless otherwise stated.

Cell volume = cell volume in cc. per cc.

The  $N_2$  taken up by the cells including internal water was arrived at as follows:

$$[N_2]_{\text{centrifugable medium}} = \alpha^\circ(1 - \text{cell volume})C_{H_2O}(p/760)$$

$$\alpha_{\text{wet cells}} = \frac{([N_2]_{\text{total}} - [N_2]_{\text{centrifugable medium}})760}{(\text{cell volume})p}$$

The  $N_2$  taken up by the dry or solid cell material was arrived at as follows:

$$[N_2]_{\text{total medium}}^* = \alpha_{\text{medium}}^{\circ} (p/760) C_{H_2O}$$

$$\alpha_{\text{dry cells}} = \frac{([N_2]_{\text{total}} - [N_2]_{\text{total medium}}) 760}{(\text{dry weight})p}$$

\* Total medium means that all the water, both inside and outside the cells, is included.

#### DISCUSSION

The results are presented in Tables I and IV and Fig. 2. The record numbers in Table IV refer to those in reference (9), where detailed physical characteristics of the experimental material are recorded. The values reported in Tables II and IV are the average of duplicate analyses unless otherwise stated.

The discrepancies between the observed and calculated volume per cent values for the lower nitrogen pressures in Tables II and III may be partly accounted for by the variable rare gas (argon) content of the gas mixtures. The deviations for air compared to pure nitrogen estimated from the argon content of air and the argon  $\alpha$  values of Estreicher (7) and Lannung (8) are approximately 0.016, 0.022, and 0.025 compared to the observed values of 0.023, 0.033, and 0.032 respectively for the three temperatures (see Tables I to IV).

The observed  $N_2$  content values reported in Table IV have been corrected by subtracting the error found in Table II, corresponding to the  $N_2$  pressure employed. In the experiments at 6.8° and 25° the error to be subtracted was estimated by the approximate relation,

$$\text{Error } t^{\circ} \approx (\text{error } 0.7^{\circ}) \times \frac{\alpha_{t^{\circ}}}{\alpha_{0.7^{\circ}}}$$

The water contents reported at 25° (Table I (9)) were always corrected to the experimental temperature by means of the water temperature coefficient of expansion, when used to calculate the values reported in Table IV.

*Solubility of  $N_2$  in Liquids and Cells Suspended in Liquids*—The values of  $\alpha_{\text{wet cells}}$  (Table IV), calculated as previously described, represent solubility coefficients that would be obtained if it were possible to work with 100 per cent cells. *The coefficient for Azoto-*



*bacter* at 0.7° was found to vary between 0.0203 and 0.0223 with an average value of 0.0213 for three different *Azotobacter* cul-

TABLE I  
*Solubility of Nitrogen in Water and in Medium at 0.7°*

Liquid	$P_{N_2}$	$N_2$ content	$H_2O$ content	$\alpha$	$\alpha^*$
	<i>mm. Hg</i>	<i>vol. per cent</i>	<i>gm. per cc.</i>	<i>cc. per cc.</i>	<i>cc. per gm. <math>H_2O</math></i>
Water	750	2.370	1.000	0.02401	0.02401
	750	2.373	1.000	2403	2403
	750	2.369	1.000	2400	2400
	750	2.371	1.000	2402	2402
	750	2.373	1.000	2403	2403
	750	2.378	1.000	2409	2409
	749	2.376	1.000	2409	2409
	749	2.378	1.000	2411	2411
Average.....				0.02405	0.02405
Medium	755	2.330	0.999	0.02348	0.02350
	755	2.322	0.999	2341	2343
	744	2.288	0.999	2338	2340
	744	2.298	0.999	2348	2350
	744	2.311	0.999	2362	2364
Average.....				0.02347	0.02349
Medium + 1% sucrose	755	2.287	0.993	0.02304	0.02320
	755	2.289	0.993	2313	2329
	754	2.276	0.993	2296	2312
	754	2.289	0.993	2309	2325
	754	2.248	0.993	2297	2313
	741	2.230	0.993	2288	2304
	741	2.258	0.993	2316	2332
Average.....				0.02303	0.02309
Medium + 0.2% sucrose	744	2.282	0.998	0.02332	0.02337
	744	2.298	0.998	2347	2352
Average.....				0.02340	0.02344

tures. At 6.8° the value of 0.0212 was obtained. The values are 93 and 106 per cent of the solubilities in the respective suspending

TABLE II  
*Nitrogen Pressure Isotherm in Water and in Medium + 1 Per Cent  
 Sucrose at 0.7°*

Liquid	$P_{N_2}$	N <sub>2</sub> content		Error, $E = (a) - (b)$
		Observed (a)	Calculated* (b)	
	mm. Hg	vol. per cent	vol. per cent	
H <sub>2</sub> O.....	749	2.377	2.371	+0.006
Medium + 1% sucrose.....	741	2.244	2.246	-0.002
H <sub>2</sub> O.....	592	1.906†	1.874	+0.032
Medium + 1% sucrose.....	586	1.818	1.786	+0.032
H <sub>2</sub> O.....	359	1.158	1.137	+0.021
Medium + 1% sucrose.....	355	1.093	1.076	+0.017
H <sub>2</sub> O.....	135	0.440	0.427	+0.013
Medium + 1% sucrose.....	134	0.426	0.406	+0.020

\* Calculated from the appropriate  $\alpha$  value in Table I.

† Single analysis.

TABLE III  
*Solubility of Nitrogen in Water at 25° and 6.8°*

Temperature	$P_{N_2}$	N <sub>2</sub> content		H <sub>2</sub> O content	$\alpha^*$	$\alpha^{**}$	Error, $E = (a) - (b)$
		Observed (a)	Calculated (b)				
°C.	mm. Hg	vol. per cent	vol. per cent	gm. per cc.	cc. per cc.	cc. per gm. H <sub>2</sub> O	
25.0	732	1.413	1.418	0.997	0.01468	0.01472	-0.005
25.0	734	1.425	1.422	0.997	1476	1480	+0.003
25.0	734	1.423	1.422	0.997	1474	1478	+0.001
Average.....					0.01473	0.01477	
25.0	579	1.144	1.122	0.997	0.01492	0.01496	+0.022
25.0	579	1.146	1.122	0.997	1495	1499	+0.024
25.0	350	0.697	0.678	0.997			+0.019
25.0	350	0.698	0.678	0.997			+0.020
25.0	350	0.687	0.678	0.997			+0.009
6.8	747	2.014		1.000	0.02050	0.02050	
6.8	747	1.996		1.000	2032	2032	
6.8	590	1.628	1.585	1.000	0.02098	0.02098	+0.043
6.8	590	1.608	1.585	1.000	2073	2073	+0.023
6.8	136	0.390	0.366	1.000			+0.024
6.8	135	0.384	0.363	1.000			+0.021

\*  $\alpha$  and  $\alpha^*$  values are reported only for essentially pure N<sub>2</sub> and for air. The other gas mixtures are arbitrary so that the  $\alpha$  values have no general interest.

TABLE IV  
*Nitrogen Taken Up by Suspensions of Azotobacter, Legume Bacteria, and Yeast*

Record No.	PN <sub>2</sub>	[N] <sub>total</sub> = N <sub>2</sub> content		$\alpha$ = N <sub>2</sub> solubility coefficient in suspension	$\alpha_{\text{wet}}$ cells = cc. N <sub>2</sub> taken up by 1 cc. wet cells per atmos- phere N <sub>2</sub> pressure	$\alpha_{\text{dry}}$ cells = cc. N <sub>2</sub> taken up by 1 gm. dry cells per atmos- phere N <sub>2</sub> pressure
		Observed	Corrected			
<i>Azotobacter</i> ; uptake at 0.7°						
46	mm. Hg	vol. per cent	vol. per cent	cc. per cc. liquid	cc. per cc. wet cells	cc. per gm. dry cells
	752	2.197*	2.197†	0.02220 ± 0.0001†	0.0203 ± 0.0003†	0.005 ± 0.002†
	594	1.765*	1.733	2219 1	202 4	5 2
	360	1.083	1.051	2220 2	203 7	5 3
	136	0.429	0.397	2220 6	202 19	5 8
Weighted averages§.....						
60	749	2.222*	2.222	0.02256 ± 0.0001	0.0216 ± 0.0003	0.009 ± 0.001
	359	1.083	1.051	2226 2	207 6	5 3
	135	0.414	0.397	2236 6	211 17	6 7
Weighted averages§.....						
64	752	2.257	2.257	0.02281 ± 0.0001	0.0223 ± 0.0003	0.012 ± 0.001
	594	1.799	1.767	2261 1	217 4	9 2
	360	1.113	1.094	2310 2	233 7	15 3
	136	0.426	0.409	2287 6	225 19	12 7
Weighted averages§.....						
0.0116.± 0.0005						

*Azotobacter*; uptake|| at 6.8°

38	740	1.952	1.952	0.02004 ± 0.0001	0.0208 ± 0.0006	0.016 ± 0.002
	585	1.571	1.544	2005 1	209 7	16 3
	355	0.970*	0.954	2042 2	228 12	24 5
	134	0.370	0.356	2020 6	211 31	19 13
Weighted average§.....				0.02012 ± 0.00003	0.0212 ± 0.0002	0.0173 ± 0.001
Heat-inactivated <i>Azotobacter</i> ; uptake   at 25°						
24-1	734	1.384*	1.384	0.01433 ± 0.0001	0.013 ± 0.004	0.004 ± 0.02
24-2	734	1.372*	1.372	1421 1	11 2	-0.005 1
24-2	357	0.706*	0.692	1472 2	21 5	0.046 2
24-3	734	1.410	1.410	1460 1	17 1	22 05
Weighted average§.....					0.015 ± 0.0005	

\* Single analysis.

† The limits of error assigned for this column are ±0.010 volume per cent.

‡ The indicated experimental error for  $\alpha_{wet}^{cells}$ ,  $\alpha_{dry}^{cells}$ , and  $\alpha$  were obtained from the error of ±0.010 volume per cent for the direct observations by use of the following factors: for  $\alpha_{wet}^{cells}$ , ±0.0001 × 760/PN<sub>2</sub> × (cell volume); for  $\alpha_{dry}^{cells}$ , ±0.0001 × 760/PN<sub>2</sub> × (dry weight); for  $\alpha$ , ±0.0001 × 760/PN<sub>2</sub>. Errors other than the solubility analytical error are considered to be included in this figure.

§ The reciprocal of the limit of error was arbitrarily assigned as the weighting of the values to be averaged. The indicated errors of the averages were likewise arbitrarily estimated by the relation  $0.6745 \sqrt{2wv^2/2w(n-1)}$  where  $w$ ,  $v$ , and  $n$  respectively represent the weighting, deviation of a single observation from the mean, and the number of observations. In some cases in which the deviations were apparently unduly small a higher error has been assigned.

|| These samples of *Azotobacter* were diluted with medium (no sucrose). The  $\alpha^\circ$  employed in making the calculations were obtained by multiplying  $\alpha_{H_2O}^\circ$  ( $^\circ$ ) by the ratio,  $\alpha_{medium}^\circ/\alpha_{H_2O}^\circ$  (0.7°); i.e.,  $\alpha_{medium}^\circ$  (6.8°) = 0.02041 (0.02349/0.02405) = 0.01994, and  $\alpha_{H_2O}^\circ$  (25°) = 0.01477 (0.02349/0.02405) = 0.01442.

TABLE IV—Concluded

Record No.	P N <sub>2</sub>	[N] <sub>2</sub> total = N <sub>2</sub> content		$\alpha$ = N <sub>2</sub> solubility coefficient in suspension	$\alpha_{\text{wet}}$ cells = cc. N <sub>2</sub> taken up by 1 cc. wet cells per atmos- phere N <sub>2</sub> pressure	$\alpha_{\text{dry}}$ cells = cc. N <sub>2</sub> taken up by 1 gm. dry cells per atmos- phere N <sub>2</sub> pressure	
		Observed	Corrected				
Legume bacteria; uptake at 0.7°							
56	mm. Hg	sol. per cent		cc. per cc. liquid	cc. per cc. wet cells	cc. per gm. dry cells	
	740	2.285	2.285	0.02345 $\pm$ 0.0001	0.0237 $\pm$ 0.0004	0.019 $\pm$ 0.001	
	585	1.840	1.808	2350	238	19	
	355	1.115	1.096	2348	236	18	
	134	0.442	0.425	2410	261	26	
Weighted averages				0.02352 $\pm$ 0.00003	0.0239 $\pm$ 0.0002	0.0190 $\pm$ 0.0004	
Yeast; uptake at 0.7°							
42-1	590	1.776*	1.751†	0.02257 $\pm$ 0.0001	0.0143 $\pm$ 0.0009	-0.0330 $\pm$ 0.004	
	590	1.721*	1.702†	2192	170	-0.0081	
	42-3	590	1.530	1.525†	1965	166	-0.0151
	43-1	578	1.767	1.735	2236	175	-0.0142
	43-2	578	1.648	1.616	2082	170	-0.0133
43-3	578	1.432	1.400	1804	170	-0.0122	
Weighted averages					0.0168 $\pm$ 0.00006	-0.0141 $\pm$ 0.0003	

\* These suspensions were made with medium and hence have been corrected by the factor  $+(2.405 - 2.347) (1 - \text{cell volume})/P/760$  to make them comparable with the water-diluted cells, as well as by the factor -0.032.

liquids and are 106 and 123 per cent of the value that might be expected on the basis of the water contents. *The coefficient for legume bacteria at 0.7°* was found to be 0.0239 or 102 per cent of the solubility in the suspending medium, and 121 per cent of the value expected on the basis of the water contents. *The coefficient for a sample of Fleischmann's yeast at 0.7°* was found to be 0.0168 or 70 per cent of the solubility (0.02405) in water and 82 per cent of the value expected on the basis of the water content.

The linear relation between  $\alpha$  and cell volume found for the yeast (compare that reported by Van Slyke, Dillon, and Margaria (17) for erythrocytes in plasma) may be expressed by the equation,

$$\alpha = 0.02385 - 0.00715 \times (\text{per cent cell volume})$$

Such a relationship was not demonstrated for *Azotobacter* and legume bacteria although, obviously, it was assumed in the calculation of  $\alpha_{\text{wet cells}}$ . It is to be noted that the high viscosity of cell suspensions of these two organisms practically prohibits solubility determination above 35 to 40 per cent cell volume. *The values of the coefficient  $\alpha_{\text{dry cells}}$ , obtained for the Azotobacter suspensions at 0.7° are 0.005, 0.008, and 0.012; at 6.8° the value 0.017 was obtained. The legume bacteria yielded the value 0.019 and yeast the negative value -0.141. Van Slyke, Dillon, and Margaria (17) obtained comparable coefficients of 0.017 and 0.019 cc. per gm. of dissolved hemoglobin at 38° and 25° respectively.*

The variation in the values of both  $\alpha_{\text{wet cells}}$  and  $\alpha_{\text{dry cells}}$  for *Azotobacter* and the change in sign with yeast may be attributed to physiological variations. Such variations would be of the nature of variable internal salt concentration, amount of "bound" water, and different proportions of cell constituents.

*Absence of Measurable Reversible Chemical Combination or Physical Adsorption of Nitrogen by Suspended Cells*—The  $\text{N}_2$  dissolved by suspensions of both *Azotobacter vinelandii* and the legume bacteria, *Rhizobium meliloti*, at 0.7° and over the pressure range from 0.18 to 1 atmosphere, was found to be directly proportional to the  $\text{N}_2$  pressure, in accordance with Henry's law (Table IV). There is no tendency for  $\text{N}_2$  content or  $\alpha$  values to be disproportionately high at low  $\text{N}_2$  pressures, as would be

$$[\text{N}_2]_{\text{content}} = \frac{k'P_{\text{N}_2}}{K_{\text{N}_2} + P_{\text{N}_2}} + \text{Volume of N}_2 \text{ taken up}$$

The upper limit of the number of molecules of  $N_2$  taken up per cell by adsorption can be calculated as follows: By analysis of the data in Table IV, especially graphically, it can be seen that the maximum value the hyperbolic function in the previous equation could have is about 0.04 cc. per 100 cc. of cells at high nitrogen pressures (saturation of  $E$ ). For Experiments 46, 60, and 64 the average gm. of dry weight per 100 cc. is 7.8, and there are about  $6 \times 10^{11}$  cells per gm. of dry weight, so that the maximum number of molecules that may be taken up per cell hyperbolically is  $0.04 (6.06 \times 10^{23}) / (22,400) (7.8) 6 \times 10^{11} = 2.31 \times 10^5$ . Similarly, the values  $4 \times 10^5$  and  $0.4 \times 10^5$  are obtained for Experiments 38 and 56. These maximum values for the nitrogen that may be taken up by *Azotobacter* cells in the prescribed hyperbolic manner may be variously expressed, approximately, as: (1) 50 per cent of the nitrogen represented by the coefficient  $\alpha_{\text{dry cells}}$ ; (2) 6 and 1 per cent respectively of the amount of nitrogen taken up by dried powdered *Azotobacter* at  $0^\circ$  and  $-183^\circ$  (see later); (3) 1 per cent of the calculated number of nitrogen molecules required to form a monomolecular layer on the external surface of cells 1 to  $2 \mu$  in diameter; (4) 0.1 per cent of the non-water molecules per cell

estimated on the basis of a diameter of  $2\ \mu$ , a density of 1.1, a water content of 76 per cent, and an average non-water molecular weight of 1000.

*Uptake of Nitrogen and Argon by Dried Azotobacter and Yeast Cells*

Fig. 2 gives the data obtained for  $N_2$  and A taken up by dried powdered and unpowered *Azotobacter* at  $0^\circ$  and  $-183^\circ$  and for

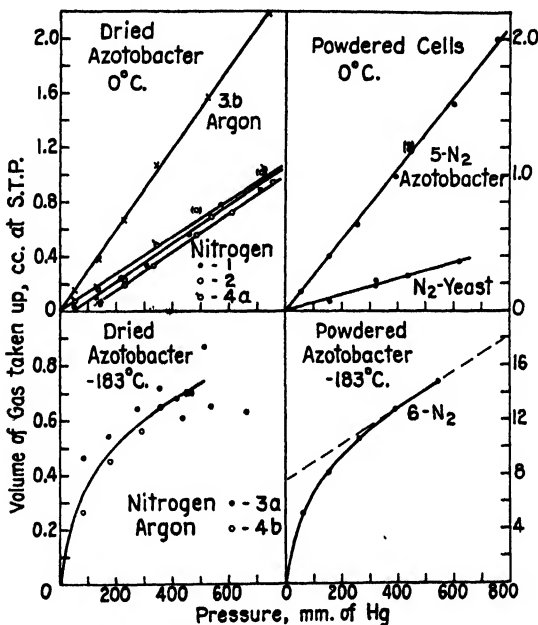


FIG. 2. The uptake of nitrogen and argon in dried powdered and unpowered *Azotobacter* and yeast cells at  $0^\circ$  and  $-183^\circ$  as a function of pressure of the gas. Nos. 1, 2, 3a, etc., give the order in which the runs were made. The points in parentheses represent values obtained either when the pressure was lowered after observation at 760 mm., or when the observation time was 18 hours. 17.4 gm. samples were used. The error is about  $\pm 0.1$  cc.; the  $0^\circ$  data are subject to the least error. No uptake great enough to observe was obtained at  $0^\circ$  with unpowered yeast.

the  $N_2$  taken up by powdered yeast. The run numbers occurring in Fig. 2 show the order in which the experiments were carried out. It is seen, therefore, that the  $N_2$  isotherm at  $0^\circ$  with un-



powdered cells is reproducible even after a run is made at  $-183^{\circ}$ . The failure of some of the curves to extrapolate to zero is attributed to experimental error, which is relatively large for the amounts of gas taken up. Powdering the dried *Azotobacter* until they appeared microscopically to be individual cells, or at most groups of four or five, resulted in a doubling of the amount of  $N_2$  taken up at  $0^{\circ}$ , but at  $-183^{\circ}$  gave a 20-fold increase in  $N_2$  uptake. Powdered yeast, reduced to individual cell size, at  $0^{\circ}$  took up one-fourth as much  $N_2$  as powdered *Azotobacter*. Unpowdered yeast was not observed to take up any gas.

As reported by Burk (3), these observations were originally believed to result from *solubility* of these gases in the dried organisms. However, while activated adsorption is readily eliminated, since argon shows the uptake as well as nitrogen, it may still be that van der Waal's adsorption is the chief factor in the uptake. Emmett, Brunauer, and Love (6) have shown that soils take up gases by van der Waal's adsorption at  $0^{\circ}$  and  $-183^{\circ}$  in the ratio 1:50, while for dried *Azotobacter* the ratio is 1:10. Thus the uptake reported here need only have a slightly different temperature coefficient than that on soils. The fact that powdering increases the uptake at  $0^{\circ}$  only 2-fold, while it increases it 20-fold at  $-183^{\circ}$ , may be consistent with either interpretation. This would require only that diffusion be limiting in a greater degree at  $-183^{\circ}$  than at  $0^{\circ}$  and to a greater degree for the unpowdered cells than for the powdered cells. It is interesting too that the ratio of the solubilities of argon and nitrogen is about that found for the uptake by dried cells at  $0^{\circ}$ , particularly when corrected for the fact that the dead space in the apparatus was determined with helium. Finally the concave downward isotherm obtained at  $-183^{\circ}$  with powdered *Azotobacter*, which according to Emmett and Brunauer (5) may be used to calculate the surface of one cell, yielded the area 3.4 to 4.1 sq.  $\mu$ . This value may be compared with the calculated surface areas 13 and 2 sq.  $\mu$  of spherical cells having diameters of 2 and 1  $\mu$  respectively.

#### SUMMARY

Typical Henry's law solubility relations were observed for nitrogen obtainable by vacuum extraction from suspensions of *Azotobacter*, legume bacteria, and yeast cells maintained at low tempera-

tures. Hyperbolic chemical or physical adsorption, of the nature suggested by the kinetics of nitrogen fixation by *Azotobacter*, was found to be beyond the limits of measurements of the present technique. Nitrogen and argon were reversibly taken up by dried *Azotobacter* and yeast cells at 0° and -183° in readily measurable quantities.

The writer is much indebted to Dr. Dean Burk for his encouragement and suggestions during the pursuit of this work. He is also indebted to Professor J. C. W. Frazer of Johns Hopkins University for suggestions and criticisms. The valuable criticisms offered by Dr. P. H. Emmett and Dr. F. E. Allison as well as Dr. Burk in the preparation of the manuscript are greatly appreciated.

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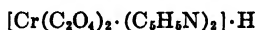
**COMPLEX SALTS OF AMINO ACIDS AND PEPTIDES**  
**III. SALTS OF DIOXALATODIPYRIDINOCHROMIATO ACID (DIOX-  
PYRIDIC ACID) AND DIOXALATODIANILINOCHROMIATO  
ACID (DIOXANILIC ACID)**

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(Received for publication, October 29, 1937)

The experiments reported in the present paper were intended to develop reagents capable of precipitating aliphatic monoamino-monocarboxylic acids. It was shown some time ago (1) that potassium trioxalatochromiate  $[\text{Cr}(\text{C}_2\text{O}_4)_3] \cdot \text{K}_3$  precipitates glycine from its solutions in dilute alcohol. It was now found that dioxalatodipyridinochromiato acid (dioxpyridic acid) (I) forms salts with amino acids



I

such as glycine, *dl*- and *l*-alanine, *l*-valine, *l*-leucine, *l*-cystine, *l*-cysteine, *dl*- and *l*-phenylalanine, *l*-tyrosine, *l*-proline, *l*-asparagine, and *l*-arginine. These salts form brilliant red crystals and are sparingly soluble in water. Lysine, aspartic acid, glutamic acid, and hydroxyproline also give dioxpyridates, some of which are of a rather complicated composition. Most of these latter salts are easily soluble and form slowly; hence there is little possibility that they interfere in the analysis of protein hydrolysates. Their detailed description may be omitted.

The dioxpyridate of alanine may be used as a means of estimating alanine. In protein hydrolysates containing glycine and alanine and only small amounts of proline, it is possible to estimate glycine with the aid of potassium trioxalatochromiate and alanine as the dioxpyridate. The method is described in detail in the subsequent paper on the structure of silk fibroin (2).

In these experiments dioxpyridic acid was employed as its

sodium salt. The sodium salt has already been prepared by Meisenheimer (3). His method was modified slightly by us in order to adapt it to large scale experiments. Sodium *trans*-dioxalatodiaquochromiate (4) was transformed, by warming with pyridine, into pyridine dioxpyridate; and this product, without being isolated, into the less soluble aniline salt. The aniline salt, on treatment with sodium acetate in methyl alcohol, gave sodium dioxpyridate.

Theoretically, a *cis* and a *trans* form of dioxpyridic acid are to be expected. Only the *trans* form is symmetrical. The *cis* form should exist in two antipodes and therefore should yield two series of salts with optically active amino acids. In no case could the existence of two series of amino acid dioxpyridates be observed. Dioxpyridic acid therefore may be regarded as having the *trans* constitution.

Owing to its great affinity to basic nitrogen compounds and to its applicability in aqueous solution, sodium dioxpyridate and the corresponding ammonium salt may become of some use for the isolation of such nitrogen compounds. The relatively high molecular weight of dioxpyridic acid and the ready crystallization of its salts favor such an application. It may be mentioned in this connection that creatine forms a rather difficultly soluble dioxpyridate.

Another complex acid that forms crystallized salts with amino acids was found to be dioxalatodianilinochromiato acid (dioxanilic acid) (II). The aniline salt of dioxanilic acid has already been prepared



## II

by Meisenheimer (3) by treating potassium *cis*-dioxalatodiaquochromiate with aniline for several weeks. Since his preparations failed to have a constant composition, he did not subject them to a closer investigation. A more favorable result was obtained when sodium *trans*-dioxalatodiaquochromiate (4) was heated with aniline to 70° for only a few hours. Two aniline salts resulted, a purple salt and a bluish green salt, both having the composition of aniline dioxalatodianilinochromiates but differing with respect to solubility and stability. It seems possible that they are *cis-trans* isomers.

Owing to the great instability of the bluish green salt, this question could not be settled.

The purple aniline salt, which is designated aniline dioxanilate, is rather difficultly soluble in water. It is transformed into the sodium salt by means of sodium acetate. This in turn may be used for the preparation of the rather stable ammonium salt. The sodium salt, as well as the ammonium salt, can be used for the preparation of amino acid salts. The dioxanilates of glycine, *l*-arginine, and *l*-tyrosine are described in the experimental section of this paper.

The bluish green aniline salt mentioned above, which was obtained as a by-product during the preparation of aniline dioxanilate, dissolves rather easily in water. In contact with a solution of ammonium chloride it is transformed into a well crystallized dichroic green-lilac salt of a complex acid containing only one aniline group and two oxalato residues. On treatment with potassium carbonate, the bluish green aniline salt splits off *both* its aniline groups; the green salt resulting under these conditions is easily obtained in crystalline form.

For the analyses reported in this paper, the author is indebted to Mr. Joseph L. Goldberg.

#### EXPERIMENTAL

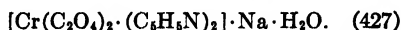
##### *Salts of Dioxypyridic Acid*

*Aniline Salt*—500 gm. of sodium *trans*-dioxalatodiaquochromiate are heated with 1 liter of pyridine on the steam bath for 3 hours, with frequent shaking. After the mixture has been cooled to room temperature, 7 liters of water and 1 liter of glacial acetic acid are added. The dark reddish liquid is filtered and 480 ml. of aniline are added. Instantaneously aniline dioxypyridate separates out in pink crystals which are filtered after several hours and washed with water. Yield, 480 gm.

[Cr(C <sub>2</sub> O <sub>4</sub> ) <sub>2</sub> · (C <sub>6</sub> H <sub>5</sub> N) <sub>2</sub> ] · NH <sub>3</sub> · C <sub>6</sub> H <sub>5</sub> .	Calculated.	C 50.0, H 3.8, N 8.8
480	Found.	" 49.9, " 3.8, " 8.7

*Sodium Salt*—500 gm. of aniline salt are stirred with a solution of 250 gm. of sodium acetate hydrate in 2 liters of methyl alcohol. Reaction takes place immediately. 250 cc. of water are added and the suspension is stirred for another hour. The sodium salt

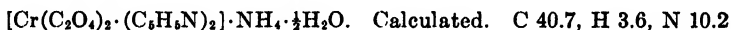
is then filtered with suction and thoroughly washed with 2 liters of methyl alcohol. The treatment with 250 gm. of sodium acetate is repeated and the product is again washed with 2 liters of methyl alcohol. When it is dried in air, the color of the salt changes from deep red to a brighter pinkish red. Yield, 380 gm. Depending upon the manner of preparation or recrystallization, the sodium salt forms different hydrates or crystals containing simultaneously methyl alcohol and water. A salt which was crystallized from water gave the following values.



Calculated. C 39.3, H 2.8, N 6.6, Na 5.4

Found. " 39.6, " 2.7, " 6.9, " 5.2

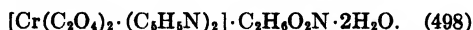
*Ammonium Salt*—10 gm. of aniline salt were treated at 0° with 100 cc. of methyl alcohol and 10 cc. of concentrated ammonia solution. After 1 hour the ammonium salt was precipitated by the addition of 100 cc. of ether. The precipitate was dissolved in a mixture of 20 cc. of water and 200 cc. of methyl alcohol and reprecipitated from the filtered solution by addition of ether. Yield, 6 gm.



413

Found. " 40.9, " 3.6, " 10.3

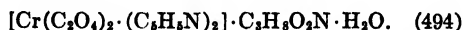
*Glycine Salt*—To a solution of 0.75 gm. of glycine in 60 cc. of water and 25 cc. of N HCl, 4.5 gm. of sodium dioxypyridate were added. Immediately, hexagonal red needles separated out. After the reaction mixture stood for several hours at 6°, the yield was 80 per cent of the theoretical.



Calculated. C 38.7, H 4.0, N 8.4,  $\text{NH}_2\text{-N}$  2.81

Found. " 38.7, " 4.0, " 8.5, " 2.89

*dl-Alanine Salt*—The preparation was similar to that of the glycine salt. Yield, 85 per cent of the theoretical.



Calculated. C 41.3, H 4.1, N 8.5,  $\text{NH}_2\text{-N}$  2.84

Found. " 41.4, " 4.2, " 8.6, " 2.81

*l-Alanine dioxypyridate* had the same composition.

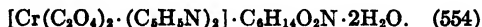
*l-Valine Salt*—Yield, 75 per cent of the theoretical.



Calculated. C 42.2, H 4.8, N 7.7,  $\text{NH}_2\text{-N}$  2.59

Found. " 42.4, " 4.8, " 7.5, " 2.51

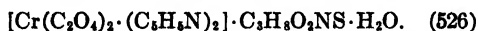
*l-Leucine Salt*—Six-sided platelets. Yield, 89 per cent of the theoretical.



Calculated. C 43.3, H 5.1, N 7.6,  $\text{NH}_2\text{-N}$  2.53

Found. " 43.4, " 5.1, " 7.9, " 2.56

*l-Cysteine Salt*—The cysteine salt formed four-sided microscopic plates of an orange-pink color. Yield, 85 per cent of the theory.



Calculated. C 38.8, H 3.8, N 8.0,  $\text{NH}_2\text{-N}$  2.66

Found. " 39.0, " 3.7, " 7.7, " 2.76

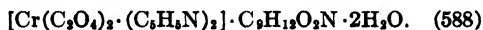
*l-Cystine Salt*—This compound forms brilliant dark red crystals possessing well built faces. Yield, 80 per cent of the theory.



Calculated. C 38.2, H 3.7, N 7.9

Found. " 38.0, " 3.7, " 7.6

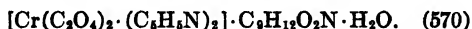
*dl-Phenylalanine Salt*—1.65 gm. of *dl*-phenylalanine were dissolved in a mixture of 20 cc. of water and 15 cc. of  $\text{N HCl}$ . After addition of 4.5 gm. of sodium dioxypyridate, the phenylalanine salt crystallized out immediately. After the material stood for several hours at  $6^\circ$ , the yield was 5.7 gm. or 96 per cent of the theory.



Calculated. C 46.9, H 4.4, N 7.1,  $\text{NH}_2\text{-N}$  2.38

Found. " 46.9, " 4.4, " 7.4, " 2.39

*l*-Phenylalanine gave a dioxypyridate that contained only 1 molecule of water of crystallization.



Calculated. C 48.4, H 4.2, N 7.4

Found. " 48.0, " 4.0, " 7.5

*l-Tyrosine Salt*—1.8 gm. of *l*-tyrosine were dissolved in 45 cc. of  $\text{N HCl}$ , and 6.6 gm. of sodium dioxypyridate were added. Octagonal, glittering plates soon separated out. After the material



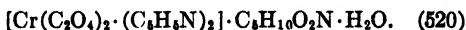
stood at 6° for 2 days, the yield was 5.7 gm. or 70 per cent of the theory. The composition of the salt was rather peculiar.



Calculated. C 44.2, H 4.1, N 6.9,  $\text{NH}_2\text{-N}$  1.71,  $\text{Na}$  0.70

Found. " 44.1, " 4.1, " 7.0, " 1.76, " 0.78

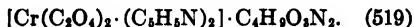
*l-Proline Salt*—Well formed plates were obtained. Yield, 80 per cent of the theory.



Calculated. C 43.8, H 4.2, N 8.1

Found. " 43.8, " 4.2, " 8.1

*l-Asparagine Salt*—The solution of 1.3 gm. of *l*-asparagine in 50 cc. of 0.5 N HCl gave red crystals instantaneously upon adding 4.5 gm. of sodium dioxypyridate. After several hours at 6°, the yield was about 72 per cent of the theory.



Calculated. C 41.6, H 3.7, N 10.8,  $\text{NH}_2\text{-N}$  2.70

Found. " 41.7, " 3.7, " 10.9, " 2.76

*l-Arginine Salt*—1.05 gm. of arginine monohydrochloride were dissolved in 25 cc. of 0.5 N HCl and 4.5 gm. of sodium dioxypyridate were added. At once crystals separated out. They were kept for several days at 6° before filtration. Yield, 4.5 gm.

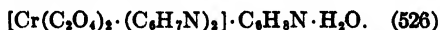


Calculated. C 39.3, H 4.4, N 10.8,  $\text{NH}_2\text{-N}$  1.35

Found. " 39.5, " 4.4, " 10.8, " 1.28

#### *Salts of Dioxanilic Acid*

*Aniline Salt*—60 gm. of sodium *trans*-dioxalatodiaquochromiate were heated with 100 cc. of aniline for 4 hours in a bath of 70°. The mixture was frequently shaken, until all was solidified. Finally it was cooled and thoroughly suspended in a mixture of 80 cc. of glacial acetic acid and 250 cc. of water. The lilac precipitate was filtered with suction and washed with ice-cold water containing a little acetic acid. Yield, 39.5 gm.



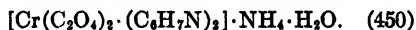
Calculated. C 50.2, H 4.6, N 8.0

Found. " 50.3, " 4.8, " 7.8

The acid mother liquor of the above preparation gave the isomeric bluish green aniline salt, discussed below.

*Sodium Salt and Ammonium Salt*—50 gm. of aniline salt were stirred with 250 cc. of methyl alcohol and 25 gm. of sodium acetate hydrate. At once transformation into purple needles took place. They were filtered after 1 hour, washed with 250 cc. of methyl alcohol, subjected to a second treatment with sodium acetate in methyl alcohol, and washed with methyl alcohol and ether. Finally, 42 gm. of sodium salt were obtained.

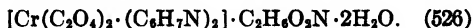
This sodium salt is not very stable and in solution it soon undergoes a spontaneous decomposition. The ammonium salt, however, is much more stable than the sodium salt; therefore, it is best to transform the sodium salt, immediately after isolation, into the ammonium salt. For this purpose the sodium salt was added to a solution of 25 gm. of ammonium chloride in 250 cc. of water. On shaking, the sodium salt went into solution and lilac needles of an ammonium salt separated out. Soon these needles started to change into purple prisms. This change was completed after a few hours. The ammonium salt was filtered and washed with ice-cold water. Yield, over 40 gm. from 50 gm. of aniline salt, or approximately quantitative.



Calculated. C 42.7, H 4.4, N 9.3, H<sub>2</sub>O 4.0

Found. " 42.8, " 4.5, " 9.1, " 4.1

*Glycine Salt*—0.75 gm. of glycine was dissolved in 20 cc. of 0.5 N HCl and 20 cc. of methyl alcohol, and 4.5 gm. of sodium dioxanilate were added. The needles of the glycine salt were filtered with suction after several hours. Yield, 4.2 gm.



Calculated. C 41.1, H 4.6, N 8.0

Found. " 41.2, " 4.5, " 8.0

*l-Tyrosine Salt*—To a solution of 0.9 gm. of *l*-tyrosine in 25 cc. of 0.2 N HCl 2.3 gm. of ammonium dioxanilate were added. Tyrosine dioxanilate crystallized out rapidly in the form of lilac prisms.

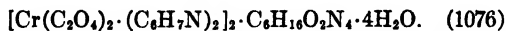


Calculated. C 47.5, H 4.7, N 6.6

Found. " 47.6, " 4.9, " 6.7

*l-Arginine Salt*—To a solution of 2.1 gm. of arginine monohydrochloride in 40 cc. of water there were added first 3.6 gm. of am-

monium dioxanilate and then, with shaking, 15 cc. of  $N$  HCl. The ammonium salt went into solution and long needles of the arginine salt crystallized out. They were filtered after a few hours and washed with water. Yield, 3.9 gm.

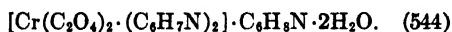


Calculated. C 42.4, H 4.8, N 10.4

Found. " 42.7, " 4.7, " 10.2

*Isomeric Aniline Dioxalatodianilinochromiate (Bluish Green Salt)*

This salt was repeatedly, but not always, obtained from the acid mother liquor from the preparation of aniline dioxanilate. By cooling this mother liquor overnight to  $6^\circ$ , flat bluish green prisms separated out, which in yellow light had a grayish lilac color. With an initial 60 gm. of sodium *trans*-dioxalatodiaquochromiate, 13.2 gm. of the bluish green aniline salt were obtained as maximal yield. The product was dried on a porous plate at  $6^\circ$  in a dark room. Due to the instability of this salt, recrystallization was not possible; the analytical data, therefore, were not very precise.



Calculated. C 48.5, H 4.8, N 7.7

Found. " 49.0, " 5.2, " 7.8

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## ON THE STRUCTURE OF SILK FIBROIN

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### *Structure of Fibroin*

The estimation of the exact number of amino acid residues contained in 1 molecule of any simple homogeneous protein has been accomplished through the use of a method recently developed in this laboratory (1, 2). It was found that the molecules of cattle globin and cattle fibrin each contained  $576 = 2^6 \times 3^2$  amino acid residues, whereas the molecule of egg albumin was found to contain  $288 = 2^5 \times 3^2$  residues. Furthermore, when the number of amino acid residues present in 1 molecule of a given protein was multiplied by the average weight of the residues, the minimum molecular weights of the above proteins were obtained. In this manner the molecular weight of egg albumin was found to be 35,700 (*i.e.*  $123.9 \times 2^5 \times 3^2$ ) which is within the limits of the range of values (31,000 to 41,000) ordinarily considered as the so called Svedberg unit. Similarly, the molecular weights of cattle fibrin and cattle globin were found to be 69,300 (*i.e.*  $120.3 \times 2^6 \times 3^2$ ) and 66,500 (*i.e.*  $115.5 \times 2^6 \times 3^2$ ) or twice the magnitude of the above Svedberg unit. Thus, in the case of egg albumin, cattle globin, and cattle fibrin it was shown that the observed molecular weights of approximately  $\nu \times 34,500$  were a direct consequence of two facts; namely, that these protein molecules contained  $\nu \times 288$  amino acid residues and that these residues had an average weight of approximately 120. Furthermore, it was concluded that those proteins whose molecular weights were  $\nu \times 34,500$  and whose constituent amino acids possessed an average residue weight of approximately 120 must contain  $\nu \times 288$  or, generally,  $2^n \times 3^m$  amino acid residues per molecule. Among the individual proteins cited as examples of the above group were insulin, pepsin,

and Bence-Jones protein. On the other hand, it was pointed out that the molecular weights of those proteins that contain a large proportion of amino acid residues of low molecular weight, *e.g.* silk fibroin and the collagens, would exhibit considerable deviation from the Svedberg unit and its multiples. Therefore, it was established for the first time that the various protein molecules fall into groups containing a definite number of units rather than into groups of definite weight, and it was concluded that the number of amino acid residues contained in any simple protein molecule was  $\nu \times 288$  or, generally,  $2^n \times 3^m$ , where  $n$  and  $m$  are positive whole numbers and  $\nu$  a whole number other than zero.<sup>1</sup>

In this investigation we have accepted the task of determining the mutual proportions of glycine, alanine, tyrosine, and arginine in a silk fibroin hydrolysate and from these results we have, wherever possible, drawn conclusions as to the general structure and the molecular weight of fibroin. New methods for the determination of glycine and alanine have been devised and are described in a subsequent paragraph.

Because of its desirable properties and its ready accessibility, silk fibroin has been used many times in attempts to gain general information on the structure of proteins. For example, in a series of studies (1906 to 1911)<sup>2</sup> on the partial hydrolysis of fibroin with mineral acids, Fischer and Abderhalden (4-9) isolated glycyl-*l*-alanine anhydride, glycyl-*l*-tyrosine anhydride, glycyl-*l*-alanine, glycyl-*l*-tyrosine, *l*-alanylglycine, *l*-alanylglycyl-*l*-tyrosine, and a non-crystalline tetrapeptide which contained 2 molecules of glycine, 1 molecule of alanine, and 1 molecule of tyrosine and which, on partial hydrolysis, yielded glycyl-*l*-alanine anhydride and glycyl-*l*-tyrosine anhydride. In recent years Abderhalden

<sup>1</sup> In a previous communication (2) the total number of residues per molecule was given as  $2^n \times 3^m$  or  $n \times 288$ . It is evident that the numerical value of the exponential  $n$  cannot be identical with that of the factorial  $n$ , and in order to emphasize this point we have replaced the factorial  $n$  by the symbol  $\nu$ . From recent investigations of Svedberg (3) it appears that the value of  $\nu$  in the expression  $\nu \times 288$  is not restricted to simple whole numbers, but in addition may be a fraction; *e.g.*,  $\frac{1}{2}$  in the case of those proteins the molecular weight of which is approximately 17,000.

<sup>2</sup> Since all natural amino acids have the same spatial configuration and belong to the so called *l* family, we designate them as *l* compounds regardless of their optical behavior.

and his coworkers claim to have isolated additional cleavage products of fibroin, but in these cases the method of characterization is not convincing. In conclusion, attention is called to the paper of Uchino (10), which contains a comprehensive bibliography on the partial hydrolysis of fibroin.

The x-ray fiber diagrams noted by Herzog and Jancke (11), Brill (12), and Kratky (13) led to the view that silk fibroin was composed of a crystalline fiber admixed with an amorphous incrusting substance. Brill (12) pointed out that the interference supposedly exhibited by the fibroin crystallite could be produced by an elementary cell in which four alanyl-glycine residues were present. Meyer and Mark (14) assumed that the crystalline portion of fibroin consisted of long peptide chains which contained glycine and alanine in alternate positions. The accessory assumption that silk fibroin contained incrusting substances in addition to a fiber crystallite can be attributed to the desire to explain the x-ray diagram on the basis of a possible elementary periodicity within the chain of the fibroin crystallite. To achieve this end the tyrosine and the other amino acids were relegated to the incrusting substance. However, as Astbury has pointed out (15), this accessory hypothesis is unnecessary as the "period" of approximately 7 Å. occurring in the direction of the fiber axis can be explained by merely assuming the presence of an extended polypeptide chain constructed of various  $\alpha$ -amino acids.

The percentages of glycine, alanine, tyrosine, and arginine which were obtained as the result of our analysis of silk fibroin are given in Column 1 of Table I, together with the earlier values for histidine and lysine which were determined by Vickery and Block (16). The percentage values were recalculated on a gm. molecular basis and are presented in this form in Column 3 of Table I. The gm. molecular ratios of the six amino acids are listed in Column 5 of Table I. The mean molecular weight of the amino acids formed by the complete hydrolysis of fibroin was estimated to be 102, which in turn leads to the value of 84 for the average residue weight. From this latter value, it was calculated that 100 gm. of silk fibroin must, on complete hydrolysis, produce approximately 1.190 gm. molecules of the hypothetical average amino acid. When one compares this value with the values for the individual amino acids given in Column 3 of Table I, it is

apparent that the amino acids glycine, alanine, tyrosine, arginine, lysine, and histidine account for  $\frac{1}{2}$ ,  $\frac{1}{4}$ ,  $1/16$ ,  $1/216$ ,  $1/648$ , and  $1/2592$  of the total number of amino acids produced by the complete hydrolysis of fibroin. From the values given in Columns 5 and 6 of Table I it follows that silk fibroin must contain  $2592 = 2^5 \times 3^4$  amino acid residues or a whole number multiple thereof, and on multiplying 2592 by the average residue weight, *i.e.* 84, it is evident that the minimum molecular weight of silk fibroin is approximately 217,700. When the value of 3.5 Å. is accepted as the distance between two adjacent peptide bonds (15) and when it is assumed that the peptide chain is situated along the fiber

TABLE I  
*Ratio of Amino Acids in Silk Fibroin after Hydrolysis*

Amino acid	Weight	Mol. wt.	Gm. molecule per 100 gm. protein		Ratio	Fraction of total residues (frequency)
			Found	Calculated*		
	(1)	(2)	(3)	(4)	(5)	(6)
	<i>per cent</i>					
Glycine.....	43.8	75	0.584 <sub>0</sub>	0.584 <sub>0</sub>	1296	2
Alanine.....	26.4	89	0.296 <sub>8</sub>	0.292 <sub>0</sub>	648	4
Tyrosine.....	13.2	181	0.072 <sub>9</sub>	0.073 <sub>0</sub>	162	16
Arginine.....	0.95	174	0.005 <sub>8</sub>	0.005 <sub>4</sub>	12	216
Lysine.....	0.25†	146	0.001 <sub>7</sub>	0.001 <sub>8</sub>	4	648
Histidine.....	0.07†	155	0.0004 <sub>8</sub>	0.0004 <sub>8</sub>	1	2592

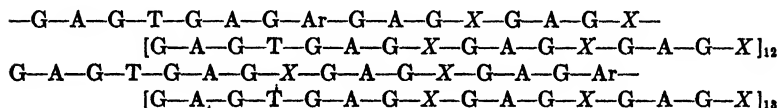
\* Base = 0.584<sub>0</sub> (gm. molecule of glycine).

† Vickery and Block (16).

axis, it follows that the length of the minimum molecule is 9.072 Å. It should be pointed out here that the percentage value of histidine which was employed in the above calculation was accepted with considerable hesitation because of the extraordinarily low histidine content of fibroin. Because of this latter fact, we have not attempted a new estimation of this amino acid, and it appears that this point cannot be settled until a more satisfactory method for the determination of histidine is devised. When all consideration of the histidine content of fibroin was omitted, it was found that the minimum number of amino acid residues in the fibroin molecule was changed to  $1292 = 2^4 \times 3^4$  and the mini-

imum molecular weight to approximately 108,500. However, it is unlikely that these latter values are correct, since if this were the case, the above histidine value would have to be in error by 100 per cent. Therefore, although a final commitment is not possible at this time, we favor the value of  $2592 = 2^5 \times 3^4$  as the number of amino acid residues in the fibroin molecule, which in turn leads to the molecular weight of approximately 217,700.

The six hydrolytic products of silk fibroin the stoichiometrical relationships of which have been determined account for approximately 85 per cent of all of the amino acid residues originally present in the intact protein. Thus, with this high percentage of cleavage products accounted for, it was possible to arrange them in a sequence which offered a rather complete structural characterization of the fibroin molecule. A fragment of this structure is given below.



(The symbols G, A, T, and Ar refer respectively to the residues of glycine, alanine, tyrosine, and arginine. The symbol *X* refers to the residues of the various other amino acids.)

The above fragmentary formula containing 432 amino acid residues is subject to a series of variations in which the frequencies of the glycine, alanine, tyrosine, and arginine residues are not altered. That is, one can withdraw one or more amino acid residues from the beginning of the chain and transfer them, in their entirety and without altering the sequence of the amino acid residues in the transferred segment, to the end of the chain. In this manner, one can construct 431 isomeric variations of the above structural fragment. One of these 432 isomers, when arranged in a linear sequence with five additional identical fragments, leads to the correct structure of the complete fibroin molecule containing 2592 amino acid residues. It should be emphasized again that in the above structural variations the sequence of the amino acid residues within the peptide chain is not altered; the only difference being that the beginning of the peptide chain is at a different position from that given in the fragment formulated above. As soon as it is experimentally possible to ascer-



tain how far distant the first arginine residue is from either end of the peptide chain of the intact fibroin molecule, then a decisive selection of one of the 432 isomers mentioned above can be made.

It is evident that the present analysis of silk fibroin demonstrates that this protein is constructed along the same general structural principles as those previously advanced for cattle fibrin, cattle globin, and egg albumin. The structural principles are described by the following equations. (1) The total number of amino acid residues in the protein molecule  $= N_i = 2^n \times 3^m$ . (2) The number of residues per molecule of protein of any one particular amino acid  $= N_i = 2^{n'} \times 3^{m'}$ . (3) The frequencies of the residues of the various individual amino acids  $= F_i = 2^{n''} \times 3^{m''}$ , where  $n$  and  $m$  are positive whole numbers;  $n'$ ,  $m'$ ,  $n''$ , and  $m''$  are either zero or positive whole numbers; and  $n = n' + n''$  and  $m = m' + m''$ . In a previous communication (17) it was suggested that the above structural principles are a consequence of the specificity of those enzymes that synthesize proteins *in vivo*. One can therefore expect that the above equations which define the general structure of the proteins investigated in this laboratory are valid also for all simple homogeneous proteins. The various individual proteins differ from one another in that the magnitude of  $n$  and  $m$  varies with the nature of the protein and because  $n'$ ,  $m'$ ,  $n''$ , and  $m''$  assume characteristic values for every protein and every protein hydrolysis product. Therefore these numerical values collectively are the essential characteristics of any protein and can be employed as the basis of a purely chemical classification of the numerous individual proteins when sufficient experimental data are made available.

It has been pointed out above that in the case of egg albumin  $N_i = 2^5 \times 3^2$ , whereas with cattle fibrin and cattle globin  $N_i = 2^6 \times 3^2$ . Silk fibroin with a minimum value of  $N_i = 2^5 \times 3^4$  is the first protein encountered in this series of investigations, in which the value of  $m$  in the expression  $N_i = 2^n \times 3^m$  is greater than 2. From the results of Svedberg's determinations on the particle size of proteins (3) it is likely that still greater values of  $n$  and  $m$  are to be found. In any event, the formula  $N_i = 2^n \times 3^m$  offers an explanation for the fact that the particle size of many simple homogeneous proteins (3) can be expressed as the product

of an *apparent* constant; *i.e.*, the Svedberg unit and a factor which is a power or a product of the numbers 2 and 3.<sup>3</sup>

In respect to the stoichiometrical composition of its molecule and in the arrangement of the amino acid residues within its molecule, silk fibroin is distinguished from all other known proteins by its simplicity of structure. The two simplest aliphatic monoaminomonocarboxylic amino acids account for three-fourths of all the amino acid residues present in the fibroin molecule; and with the glycine residues exhibiting a frequency of 2 and the alanine residues a frequency of 4, the spatial configuration of silk fibroin may be represented by the following diagram. (In the

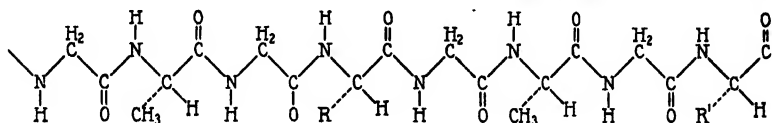


diagram the hydrogen atoms linked to the asymmetric carbon atoms are pictured as being in front of the plane of the paper and the groups  $\text{CH}_3$ , R, and  $\text{R}'$  behind the plane of the paper.) In this diagram the "backbone" of the peptide chain was constructed according to a scheme previously proposed by Meyer and Mark (14) and Astbury (15). If this formulation is correct, it becomes apparent, from the above formula, that *all* of the asymmetric carbon atoms and the side chains attached to these asymmetric

<sup>3</sup> It should be pointed out that in this and preceding discussions (1, 2, 17) we have restricted ourselves to the consideration of simple homogeneous proteins. On the other hand, it is probable that there are proteins which are truly reversible association products of two or more simple molecular species (3). In such cases, if one considers the association product as a single molecular species, then it is possible that prime numbers other than 2 and 3 may appear in the above factor. For example, if a particle, which is stable throughout a certain concentration and pH range, is composed of 1 molecule containing  $864 = 2^5 \times 3^3$  amino acid residues and a 2nd molecule containing  $576 = 2^6 \times 3^2$  residues, then the total number of residues in the particle,  $N_t = 1440 = 2^5 \times 3^2 \times 5 = 288 \times 5$ , and the apparent molecular weight, if the average weight of all the residues is equal to 120, will be 172,800 or  $5 \times 34,560$ . In such cases the particle of 1440 units should not be considered as a simple homogeneous molecule but rather as a mixture of two simple proteins which in turn are defined by the stoichiometrical laws previously described.

carbon atoms will lie on *one* side of the main chain. This phenomenon has been observed in no other protein, and its existence places fibroin in a unique position with respect to the other natural proteins.

Although no protein other than fibroin has been found to contain a monoaminomonocarboxylic amino acid exhibiting the high frequency of 2, the scleroproteins collagen and elastin have been shown to contain sufficient glycine (18, 19) to permit this amino acid to be present as every third residue; *i.e.*, a frequency of 3. Thus the proteins fibroin, collagen, and elastin have the common property of containing greater equivalent amounts of aliphatic monoaminomonocarboxylic amino acids than are possessed by all of the other known proteins. On the other hand, when silk fibroin is compared with another group of scleroproteins, *i.e.* the keratins, there is little similarity in respect to the stoichiometrical composition of their molecules.

Another striking characteristic of the fibroin molecule is the extraordinarily low content of basic amino acid residues. The question has repeatedly been brought up as to whether or not substances of such extreme composition as silk fibroin should be classified as genuine proteins. From the foregoing discussion in this paper it is evident that silk fibroin conforms to the same general stoichiometrical laws as those that govern the structure of recognized proteins.

#### *Estimation of Glycine and Alanine*

The general procedure employed for the estimation of glycine consisted of the precipitation of this amino acid with potassium trioxalatochromiate (20) and the subsequent estimation of the amino nitrogen content of the precipitate. After the optimum experimental conditions for the precipitation of glycine with potassium trioxalatochromiate had been ascertained, a solution of known glycine content was treated with the reagent under the most desirable experimental conditions in order to determine what per cent of the total glycine was precipitated in the form of the complex. The results of four determinations, each conducted in duplicate, agreed within  $\pm 1$  per cent and the average recovery of glycine from the solution was 88 per cent of the amount originally present. Analogous precipitation experiments were conducted

with the use of a solution which contained glycine, alanine, and tyrosine in approximately the same concentrations as were present in a fibroin hydrolysate. The recoveries in this case were identical, within experimental limits, with those obtained with a solution of pure glycine. From the above information the necessary correction factors were evaluated; and from the knowledge of the amount of glycine precipitated by potassium trioxalatochromiate from a definite quantity of fibroin hydrolysate, the glycine content of silk fibroin was readily ascertained.

For the determination of alanine a fibroin hydrolysate that had been freed of the major portion of glycine with the aid of potas-

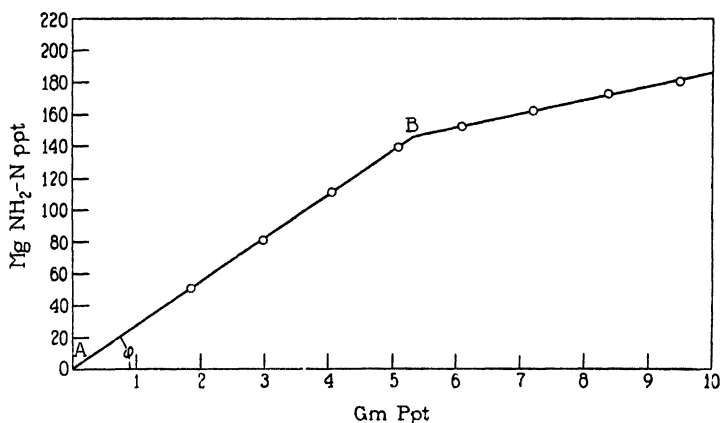


FIG. 1

sium trioxalatochromiate was subjected to a fractional precipitation with sodium dioxypyridate (21). The general procedure and the interpretation of the experimental results are as follows: Equal amounts of the solution under investigation were treated with increasing amounts of sodium dioxypyridate under identical experimental conditions. With very small amounts of the reagent no precipitation occurred, but after a certain point with increasing quantities of the reagent correspondingly increasing amounts of a precipitate were obtained. The individual precipitates were weighed and their amino nitrogen content was determined. The results thus obtained are graphically presented in Fig. 1, in which the weight of the precipitate was plotted against

the amount (in mg.) of amino nitrogen contained in the precipitate. On examination of Fig. 1 it is seen that the experimental values at first lie on the line  $AB$  which passes through the 0 point of the coordinate system. However, as soon as the amount of precipitate exceeds a certain value, which in the case investigated was 5.37 gm., corresponding to point  $B$  in Fig. 1, the amino nitrogen content of the precipitate is altered. Between points  $A$  and  $B$  the amino nitrogen content of the precipitates is approximately equal to the value of  $\tan \varphi$  or 2.75 per cent. There are only two naturally occurring amino acids that yield dioxypyridates having an amino nitrogen content in the neighborhood of the above value: they are glycine dioxypyridate with an amino nitrogen content of 2.81 per cent, and alanine dioxypyridate with 2.83 per cent. Despite the fact that glycine was ruled out of consideration for various reasons, it was decided to obtain definite proof that the precipitate corresponding to any point along the line  $AB$  was pure alanine dioxypyridate. Therefore a preparation corresponding to point  $B$  in Fig. 1 was subjected to a comprehensive analysis and it was found that with respect to its content of carbon, hydrogen, total nitrogen, and amino nitrogen it agreed perfectly with the theoretical values required by alanine dioxypyridate. In addition, the amino acid was recovered from its dioxypyridate and characterized as the *p*-toluosulfo derivative. The properties of this derivative corresponded in every respect to those previously reported for *p*-toluosulfo-*l*-alanine (22).<sup>2</sup>

It should be pointed out that the amount of alanine estimated from Fig. 1 is that precipitated as pure alanine dioxypyridate from the solution under investigation. Because of the solubility of alanine dioxypyridate, under the experimental conditions employed, a fixed amount of alanine escapes precipitation. In order to ascertain the magnitude of this loss, a second plotting of the experimental results was made, as shown in Fig. 2 where the amount (in mg.) of amino nitrogen precipitated from the solution was plotted against the amount of sodium dioxypyridate employed for the precipitation. As in Fig. 1, the values representing the amount of pure alanine dioxypyridate precipitated fell on the line  $AB$ , which in this case cut the abscissa at point  $A$ . The distance of  $A$  from the origin of the system of coordinates corresponds to the amount of sodium dioxypyridate which must be pres-

ent in solution before any alanine dioxypyridate can be precipitated. From the distance of either *A* or *D* from the origin of the system of coordinates one can readily calculate the quantity of alanine remaining in solution as the dioxypyridate. When this value is added to the amount isolated as pure crystalline alanine dioxypyridate, the total alanine content of the solution under investigation is obtained. In this manner the alanine content of silk fibroin was estimated to be 26.4 per cent.

The above procedure of fractional precipitation and its subsequent graphical interpretation appear to be of general use for many different reagents. In this laboratory this method has

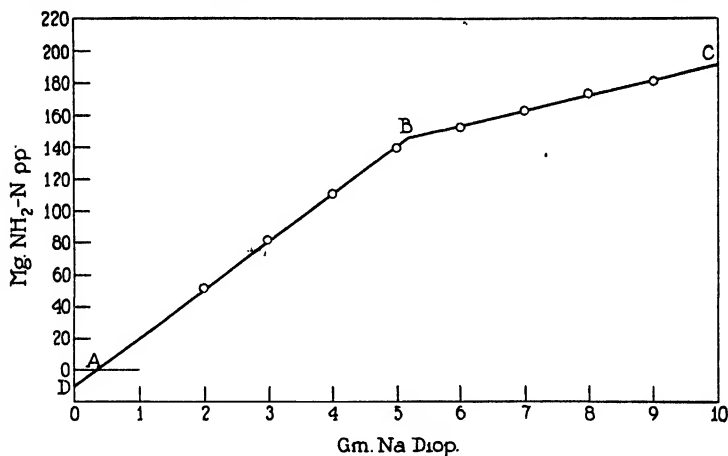


FIG. 2

been applied not only to sodium dioxypyridate but also to ammonium rhodanilate.<sup>4</sup> The application to other reagents is obvious.

#### *Digestion of Silk Fibroin with Papain*

It has been known for some time (23) that proteins are soluble in a solution of ammoniacal copper hydroxide and that they can be precipitated from such a solution by acidification. When

<sup>4</sup> In the case of ammonium rhodanilate the application of this general method has been of value in the exact determination of proline, and it has been found that various commercial samples of gelatin and a preparation of collagen contain from 14.5 to 16.5 per cent of proline.

fibroin was treated in this manner, it acquired the property of being digestible by the enzyme papain.<sup>5</sup> The digestion of this modified silk fibroin with papain-HCN proceeds so far that approximately 60 per cent of all of the peptide bonds are split.<sup>6</sup> In addition, it was found that more than three-fourths of the total tyrosine, approximately one-half of the total glycine, and more than one-fourth of the total alanine were present in the fibroin digest as the free amino acid (Table II). This fact offers further support for the suggestion (24) that the action of the proteinases (endopeptidases) is not limited to the formation of peptides of high molecular weight, but is responsible also for the formation of free amino acids. The case of silk fibroin is particularly striking as every second residue in the molecule is a glycine residue. Since approximately one-half of the glycine is liberated as

TABLE II  
*Isolation of Amino Acids from Enzymatic Digest*

Amino acid	Protein (1)	Isolated (2)	Yield (3)
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Glycine .....	43.8	18.0	41.1
Alanine.....	26.4	6.5	24.6
Tyrosine.....	13.2	10.3	78.0

the free amino acid, it is very likely that the digestion products, other than the free amino acids, are, for the greater part, peptides of very low molecular weight.

In conclusion, we wish to thank Mr. J. L. Goldberg and Mr. W. P. Anslow for their valuable assistance in this investigation.

#### EXPERIMENTAL

*Preparation of Silk Fibroin*—The protein was prepared, according to the procedure of Fischer and Skita (25), from technically degummed silk (*Bombyx mori*, L.) of Japanese origin.

*Preparation of Acid Hydrolysate*—500 gm. of air-dry fibroin

<sup>5</sup> Unpublished experiments of Dr. J. S. Fruton have shown that pancreatin can also digest this modified fibroin.

<sup>6</sup> When blood fibrin is digested with papain-HCN, approximately 64 per cent of the peptide bonds are hydrolyzed.

were refluxed with 2 liters of concentrated hydrochloric acid for 12 hours and the filtered hydrolysate was subjected to repeated vacuum distillations in order to remove the excess hydrochloric acid. The concentrate was then made up to 2 liters. This solution was equivalent to 402.8 gm. of protein containing 19.0 per cent of nitrogen (16).

*l-Tyrosine*<sup>2</sup>—1400 cc. of hydrolysate (282 gm. of protein) were adjusted to pH 2.0 with silver oxide and after the silver chloride had been removed and washed the filtrate was concentrated to 600 cc. After the material had stood at 6° for 4 days, 15.1 gm. of tyrosine had crystallized from the hydrolysate.

$C_9H_{11}O_3N$ . Calculated, N 7.7; found, N 7.7

The filtrate was made up to 1 liter and the tyrosine content of this filtrate (Filtrate A) was estimated spectrographically to be 2.2 per cent.<sup>7</sup> Therefore, the total yield of tyrosine was 37.1 gm. or 13.2 per cent of the protein.

*l-Arginine*<sup>2</sup>—300 cc. of Filtrate A corresponding to 84.6 gm. of fibroin were diluted to 600 cc. and treated with 4.0 gm. of flavianic acid in 100 cc. of water. The crude arginine flavianate was recrystallized from water and dried to constant weight at 105° (26). Yield, 2.23 gm.

$(C_{16}H_{18}N_2SO_8) \cdot (C_6H_{14}O_2N_4)$ . Calculated, N 17.2; found, N 17.3

When correction is made for the solubility of arginine flavianate in 200 cc. of solvent, the yield is raised to 2.27 gm., which is equivalent to 809 mg. of arginine or 0.95 per cent of the protein.

*Glycine*—5 cc. of 10 per cent hydrochloric acid and 35 cc. of absolute ethanol were added to 9.0 gm. of potassium trioxalatochromiate (20) in 20 cc. of a 5 per cent glycine solution which was 0.5 N in hydrochloric acid. The reaction mixture was shaken until the transformation was complete (about 6 to 8 hours), and then kept at 6° overnight. The precipitate was recovered on a sintered glass filter and washed with 25 cc. of a cold solution containing 3 parts of absolute ethanol and 1 part of 0.5 N hydrochloric acid. The precipitate was then dried and the glycine compound was washed through the filter with water. The filtrate

<sup>7</sup> We are indebted to Dr. G. I. Lavin for this determination.



was made up to 500 cc. and the amino nitrogen content of this solution was determined in the Van Slyke apparatus. A series of four independent precipitations yielded recoveries of 88 per cent  $\pm 1$  per cent. In a second series of experiments the 5.0 per cent glycine solution was replaced by a *N* hydrochloric acid solution containing 5.0 per cent of glycine, 3.0 per cent of alanine, and 1.2 per cent of tyrosine. The recoveries in this case were identical with those of the first experiment.

500 cc. of Filtrate A (141 gm. of protein) were made up to 1 liter with 0.05 *N* hydrochloric acid. 100 cc. of this Solution B were equivalent to 14.1 gm. of protein. 5 cc. of 10 per cent hydrochloric acid and 35 cc. of absolute ethanol were added to 10 gm. of potassium trioxalatochromiate in 20 cc. of Solution B. The reaction mixture was shaken for 8 hours and then kept at 6° for 48 hours. The precipitate was recovered on a sintered glass filter and washed with 25 cc. of a cold solution containing 3 parts of absolute ethanol and 1 part of 0.5 *N* hydrochloric acid. After removal of the residual solvent, the glycine complex was washed through the filter with water and the filtrate was made up to 500 cc. Amino nitrogen was determined on a 2 cc. aliquot of the above glycine complex solution and the latter was found to contain 204.2 mg. of amino nitrogen. When correction is made for the partial recovery of glycine under the above conditions, the yield is raised to 232.0 mg. of amino nitrogen or 1243.3 mg. of glycine or 44.1 per cent of the protein. A second estimation gave 43.5 per cent, and a third 43.6 per cent. The mean of the above values, *i.e.* 43.8 per cent, was taken to be the glycine content of silk fibroin.

10 gm. of a glycine-potassium trioxalatochromiate precipitate that had been prepared from a fibroin hydrolysate according to the above procedure were dissolved in 20 cc. of water. After the addition of 22 cc. of *N* sodium hydroxide the solution was cooled in an ice-salt bath and treated with 3.75 gm. of carbobenzyloxychloride and 22 cc. of *N* sodium hydroxide. After the reaction had ceased, the solution was acidified and the precipitate was collected and dried on clay. The yield was 68 per cent of the theoretical amount based on the amino nitrogen content of the original glycine-potassium trioxalatochromiate precipitate.

The substance after recrystallization from ethyl acetate and chloroform melted at 121–122°.

$C_{10}H_{11}O_4N$ . Calculated, N 6.7; found, N 6.7

The yield of carbobenzyglycine in the above experiment compares favorably with the 72 per cent which Bergmann and Zervas obtained with the use of pure glycine (27).

*l-Alanine*<sup>2</sup>—700 cc. of Solution B were treated with 350 gm. of potassium trioxalatochromiate, 175 cc. of 10 per cent hydrochloric acid, and 1225 cc. of absolute ethanol. The reaction mixture was stirred for 6 hours and then kept at 6° overnight. The precipitate was recovered on a filter and washed with 875 cc. of the alcoholic hydrochloric acid wash solution. The filtrate and washings were combined and concentrated *in vacuo* to a syrup. The concentrate was then made up to 500 cc. with water (Solution C). Nine 20 cc. aliquots of Solution C were introduced into a series of test-tubes containing 10 cc. of water, 15 cc. of 10 per cent hydrochloric acid, and 2, 3, 4, 5, 6, 7, 8, 9, and 10 gm. of sodium dioxypyridate (21). After each test-tube had been seeded with a crystal of pure alanine dioxypyridate (21), it was shaken at room temperature for 6 hours and then kept at 6° for 48 hours. The crystalline precipitates were collected on sintered glass filters and dried to constant weight on clay at room temperature. The data yielded by these experiments are given in Column 2 of Table III. 1.000 gm. samples of each of the nine precipitates were dissolved in water and made up to 50 cc. Duplicate 2 cc. aliquots of these solutions were withdrawn for the determination of amino nitrogen. The values thus obtained are presented in Columns 3 and 4 of Table III. In order to facilitate the interpretation of the above experimental results the amount (in mg.) of amino nitrogen precipitated from the solution was plotted against the weights (in gm.) of the precipitates (Fig. 1). As the amino nitrogen contents (in per cent) of the precipitates between points A and B were constant and were approximately equal to the value calculated for pure alanine dioxypyridate, it was concluded that the amount of precipitate corresponding to point B in Fig. 1 (5.37 gm.) represented the maximum yield of pure alanine dioxypyridate obtainable under the above experimental conditions. In order to ascertain the amount of alanine dioxypyridate remaining in solution, the amount of

amino nitrogen (in mg.) precipitated from the solution was plotted as a function of the amount of sodium dioxypyridate employed in the precipitation (Fig. 2). In this graph the straight line *AB*, again representing the amount of pure alanine dioxypyridate, cuts the abscissa at point *A* and the ordinate at point *D*. The distance between *A* and the origin of the system of coordinates represents the amount (in gm.) of sodium dioxypyridate required to precipitate an infinitesimal quantity of alanine dioxypyridate from the solution under investigation, and the distance between *D* and the origin of the system of coordinates represents the amount (in mg. of amino nitrogen) of alanine re-

TABLE III  
*Precipitation of Alanine Dioxypyridate*

Na dioxypyridate (1)	Ppt. (2)	NH <sub>2</sub> -N in ppt. (3)	NH <sub>2</sub> -N ppt. (4)
gm.	gm.	per cent	mg.
2	1.84	2.77	51.0
3	2.98	2.73	81.4
4	4.05	2.73	110.6
5	5.10	2.74	139.7
6	6.11	2.50	152.8
7	7.18	2.27	163.0
8	8.38	2.07	173.5
9	9.49	1.91	181.3
10	10.72	1.88	201.5

maining in solution as alanine dioxypyridate. Thus, from the above graphical analysis it was found that 145.5 mg. of amino nitrogen were precipitated from the solution in the form of alanine dioxypyridate and, in addition to this quantity, an amount of alanine equivalent to 10.0 mg. of amino nitrogen escaped precipitation because of the solubility of alanine dioxypyridate under the above conditions. Therefore, it was concluded that 155.5 mg. of amino nitrogen were present as alanine in 20 cc. of Solution C. 155.5 mg. of amino nitrogen are equivalent to 988.6 mg. of alanine or 25.0 per cent of the protein. Because of the inherent losses incurred during the above preliminary small scale precipitations, 160 cc. of Solution C were treated with 42.0 gm. of sodium dioxypyridate (corresponding to point *B* in Fig. 2), 80 cc. of water, and

120 cc. of 10 per cent hydrochloric acid. After the procedure described above had been followed, 43.10 gm. of alanine dioxypyridate were recovered.

$C_{17}H_{20}O_{11}N_2Cr$ .	Calculated.	C 41.3,	H 4.1,	N 8.5,	$NH_2-N$ 2.8
	Found.	" 41.3,	" 4.1,	" 8.7,	" 2.8

43.10 gm. of alanine dioxypyridate are equivalent to 7.765 gm. of alanine, and after correction is made for the solubility of alanine dioxypyridate, the yield is raised to 8.353 gm. or 26.4 per cent of the protein.

40.0 gm. of the above alanine dioxypyridate were suspended in 200 cc. of water and treated with 20 gm. of barium chloride in 100 cc. of water. The reaction mixture was shaken for 4 hours and after it stood at 6° overnight, the precipitate was removed. The filtrate was freed of inorganic ions and concentrated to about 100 cc. 85 cc. of 10 per cent sodium hydroxide and 20 gm. of *p*-toluosulfochloride were added to the concentrate, and the reaction mixture was maintained between 40–50° until all of the chloride had dissolved. The solution was allowed to stand overnight and was then filtered. The filtrate was acidified with hydrochloric acid and after the reaction mixture stood at 6° for several hours, the crystalline precipitate was collected and dried on clay at room temperature. The yield was 10.0 gm. M.p., 135–136°.

$C_{10}H_{13}O_4SN$ .	Calculated,	N 5.8;	found, N 5.8
	$[\alpha]_D^{25} = -6.8^\circ$	$(c = 14.7\%$	in ethanol)

Fischer and Lipschitz (22) reported a melting point of 134–135° and a specific rotation of  $-6.8^\circ$  for *p*-toluosulfo-*l*-alanine.<sup>2</sup>

*Modification of Silk Fibroin* (28)—200 gm. of cupric hydroxide were dissolved in 554 cc. of 28 per cent ammonia and 1500 cc. of water and the resulting solution was cooled to  $-5^\circ$ . 250 gm. of air-dry technically degummed silk were dissolved in the chilled copper-ammonia reagent and the mixture was shaken on the machine for 15 minutes. The solution was then filtered through a sintered glass filter and the filtrate was acidified with acetic acid. The entire operation was accomplished within 30 minutes and the temperature of the solution was never over 5°. The precipitated fibroin was recovered from the mother liquor and washed with 2 per cent acetic acid and then with water until the wash

liquor was free of copper and of acid. The product was then run through a meat grinder, collected on a filter, and again washed with water. The porous mass was sucked on the filter until its weight was 880 gm. The yield, calculated on a dry basis, was practically quantitative.

*Digestion of Modified Fibroin by Papain-HCN*—1760 gm. of modified fibroin pulp (403 gm. of protein) were added to 1000 cc. of citrate buffer (pH 5.0), 12 gm. of potassium cyanide in 100 cc. of water, 16 gm. of finely ground papain, 2140 cc. of water, and 25 cc. of toluene. The mixture was placed in an incubator held at 37° and digested for 6 weeks. After the digestion had ceased, the hydrolysate was kept at 6° for 3 weeks. The insoluble matter was recovered on a filter and washed with 1 liter of water. The filtrate and washings were combined and the digest (5150 cc.) was stored at 6° until needed. 1 cc. of the digest contained 8.61 mg. of amino nitrogen and after hydrolysis with 20 per cent hydrochloric acid for 24 hours it contained 14.26 mg. of amino nitrogen. Thus, approximately 60 per cent of the peptide bonds were split during the enzymatic digestion.

*l-Tyrosine*<sup>2</sup>—The insoluble matter obtained from the enzymatic digestion was extracted with hot water and 39.0 gm. of tyrosine were obtained.

$C_9H_{11}O_3N$ . Calculated, N 7.7; found, N 7.7

When correction is made for the solubility of tyrosine in the original mother liquor, the yield is raised to 41.5 gm. or 10.3 per cent of the protein.

*Glycine*—30 gm. of potassium trioxalatochromiate were dissolved in 320 cc. of digest and the solution was adjusted to pH 3.0 with 10 per cent hydrochloric acid. 100 cc. of N hydrochloric acid and 800 cc. of ethanol were added to the adjusted solution, which was then stirred for 4 hours. After the reaction mixture had stood at 6° for 24 hours, the precipitate was collected on a filter, washed with alcoholic hydrochloric acid, and dried on clay at room temperature. The yield was 29.0 gm. 0.7478 gm. of the precipitate was dissolved in 50 cc. of water and 0.5 cc. aliquots of this solution were used for the determination of amino nitrogen. It was found that 1.00 gm. of the precipitate contained 26.86 mg. of amino nitrogen. Therefore, the 320 cc. of digest contained,

as a minimum value, 778.9 mg. of amino nitrogen present as free glycine. From this it follows that the original digest contained 12.535 gm. of glycine amino nitrogen or 65.1 gm. of glycine. As the recovery of glycine under the most favorable conditions is approximately 90 per cent of the amount actually present, it was concluded that the enzymatic digest contained, as a minimum quantity, 72.3 gm. of glycine or 18 per cent of the weight of the protein.

10.0 gm. of the glycine-potassium trioxalatochromiate precipitate were dissolved in 20 cc. of water and after the addition of 22 cc. of *N* sodium hydroxide the solution was cooled to  $-5^{\circ}$ . 3.75 gm. of carbobenzoxychloride and 22 cc. of *N* sodium hydroxide were added to the chilled solution and after the reaction had ceased, the reaction mixture was acidified with dilute hydrochloric acid. The crystalline precipitate was collected and dried on clay at room temperature. The yield was 2.50 gm. or 68 per cent of the theoretical amount based on the amino nitrogen content of the glycine-potassium trioxalatochromiate precipitate. After recrystallization from ethyl acetate and chloroform the carbobenzoxyglycine melted at  $121-122^{\circ}$ .

$C_{10}H_{11}O_4N$ . Calculated, N 6.7; found, N 6.7

*l-Alanine*<sup>2</sup>—200 cc. of 10 per cent hydrochloric acid were added to a solution of 60 gm. of sodium dioxypyridate in 500 cc. of the enzymatic digest (39.1 gm. of protein). The reaction mixture was seeded with a crystal of pure alanine dioxypyridate and then stirred for 4 hours at room temperature. The crystalline precipitate (rhombic system) was collected on a filter, washed with 50 cc. of cold water, and dried on clay at room temperature. The yield was 14.0 gm. A second experiment gave 13.4 gm.

$C_{17}H_{20}O_{11}N_3Cr$ . Calculated. C 41.3, H 4.1,  $NH_2-N$  2.8  
Found. " 41.4, " 4.1, " 2.8

14.0 gm. of alanine dioxypyridate are equivalent to 2.522 gm. of alanine or 6.5 per cent of the protein.

20.0 gm. of the above alanine dioxypyridate were suspended in 50 cc. of water and treated with 10 gm. of barium chloride in 50 cc. of water. After removal of the barium dioxypyridate, the filtrate was freed of inorganic ions and concentrated to approximately 50 cc. The concentrate was added to 45 cc. of 10 per cent

sodium hydroxide and 10.0 gm. of *p*-toluosulfochloride, and the acylation was conducted in the usual manner. The *p*-toluosulfo-*l*-alanine recovered from the reaction mixture melted at 135–136° and possessed the correct composition.

$C_{10}H_{13}O_4SN$ . Calculated, N 5.8; found, N 5.8

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# OXIDATION METHOD FOR BILIRUBIN DETERMINATIONS IN BILE AND MECONIUM WITH THE PHOTO- ELECTRIC COLORIMETER\*

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In this paper is presented a comparison of three oxidizing reagents at present in use for the determination of bilirubin in bile, as well as a method, in which one of the oxidizing reagents is used, for the determination of bilirubin in bile, infant's stool, and meconium with the photoelectric colorimeter (1).

An oxidation method is generally considered to be more accurate than a diazo method for the determination of bilirubin in bile and meconium, on account of the oxidation products which may already be present.

*Spectrophotometric Studies*—The spectrophotometric absorption curves of the colors resulting from the action of oxidizing reagent upon bilirubin are shown in Fig. 1. The successive changes from yellow to blue through yellow-green, green, and blue-green show no stable curve until the blue color is attained. The blue color, however, remains stable at room temperature for a considerable time before the onset of the terminal stages of oxidation.

The occurrence of an isobestic point (at  $522\text{ m}\mu$ ) supports the contention of Peterman and Cooley (2) that the green color of so called biliverdin is in reality a mixture of yellow bilirubin and blue bilicyanin in varying proportions.

In the present method measurements of light absorption are made in the photoelectric colorimeter with a red filter (Curve F, Fig. 1) which transmits light only in the region of maximum absorption of the stable blue color. The use of such a filter not only

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greatly increases the sensitivity of the method, but also eliminates the interfering effect of extraneous yellow and brown pigments

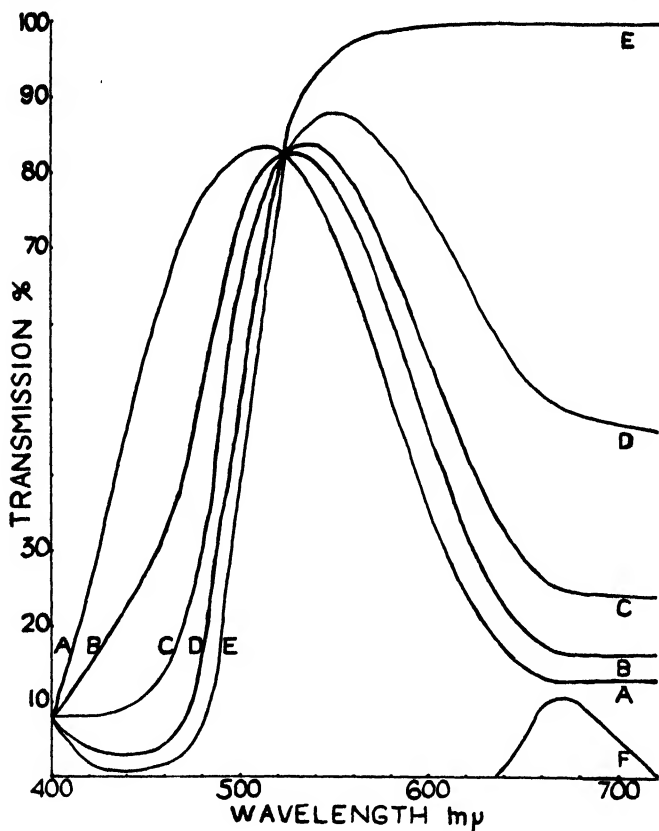


FIG. 1. Spectrophotometric curves of bilirubin oxidation products. Curve E, bilirubin alone. Curves A to D, bilirubin plus oxidizing reagent. Curve D, yellow-green, 2 minutes after addition of reagent; Curve C, green, 5 minutes; Curve B, blue-green, 10 minutes; and Curve A, blue, 20 to 200 minutes. Curve F, Filter 660. These curves were made with a nitric acid oxidizing reagent, but exactly the same series of colors is obtained with hydrogen peroxide, although the time relations of the color changes vary greatly with both the nature and concentration of the oxidizing reagent. The terminal purple and yellow colors are not shown.

whose absorption at the red end of the spectrum is negligible. These pigments are, however, a serious source of error in visual

measurements on stool, since they convert the blue color into various shades of green which cannot be matched against an artificial standard.

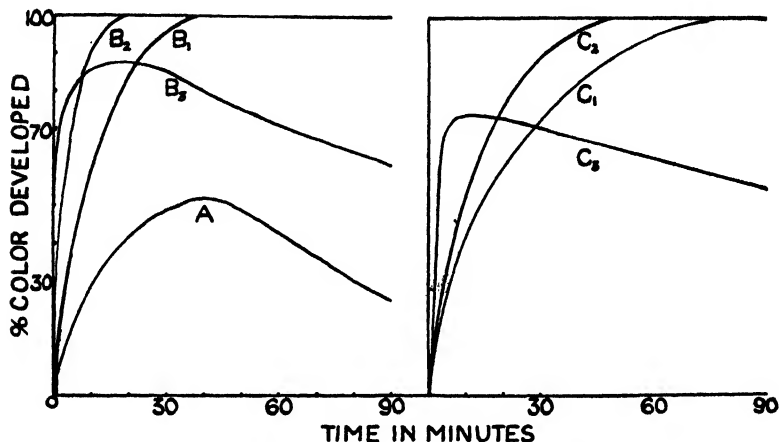


FIG. 2. Influence of nature and concentration of oxidizing reagent on rate of development and stability of blue color. Bilirubin concentration, 2.0 micrograms in 10 cc. Curve A, 2 cc. of bilirubin solution, 7.5 cc. of 1:1 glacial acetic acid-94 per cent ethyl alcohol, 0.5 cc. of ammonium persulfate. This reagent is a slight modification of that used by Schmidt, Jones, and Ivy (3). Curves B<sub>1</sub>, B<sub>2</sub>, B<sub>3</sub>, 2 cc. of bilirubin solution, 3 cc. of alcohol, 5 cc. of reagent. Reagent B<sub>1</sub>, 0.2 cc. of 30 per cent H<sub>2</sub>O<sub>2</sub>, 1 cc. of concentrated HCl in 100 cc. of 94 per cent ethyl alcohol. Reagent B<sub>2</sub>, twice the strength of B<sub>1</sub>; Reagent B<sub>3</sub>, 4 times the strength of B<sub>1</sub>. Reagent B<sub>2</sub> yields conditions almost identical with those recommended by Peterman and Cooley (2). Curves C<sub>1</sub>, C<sub>2</sub>, C<sub>3</sub>, 2 cc. of bilirubin solution, 3 cc. of alcohol, 5 cc. of reagent. In each case the reagent was allowed to stand for 1 week before being used. Fresh nitric acid reagents are much slower in their action. Reagent C<sub>1</sub>, 0.4 cc. of concentrated HNO<sub>3</sub>, 2 cc. of concentrated HCl in 100 cc. of 94 per cent ethyl alcohol; Reagent C<sub>2</sub>, twice the strength of C<sub>1</sub>; Reagent C<sub>3</sub>, 4 times the strength of C<sub>1</sub>. Reagent C<sub>2</sub> yields conditions almost identical with those recommended by Sribhishaj, Hawkins, and Whipple (4).

*Choice of Oxidizing Reagent*—Fig. 2 shows the effect of three different oxidizing reagents upon the development of the blue color, measured in the photoelectric colorimeter with Filter 660.<sup>1</sup>

<sup>1</sup>This filter is a regular accessory of the photoelectric colorimeter as sold by the Rubicon Company, 29 North Sixth Street, Philadelphia.

The ammonium persulfate reagent (Curve A), originally used for the measurement of the terminal yellow color of bilirubin oxidation (3), is plainly unsuitable for measurement of the blue color. The initial stages of oxidation are unduly prolonged, while the terminal stages proceed so rapidly that the blue color is only transient. It is, however, a source of satisfaction to note that such widely different oxidizing reagents as hydrogen peroxide and concentrated nitric acid produce the same amounts of stable blue color at their optimum concentrations. We have chosen the hydrogen peroxide reagent for the present method on account of its greater rapidity of color production, especially in determinations on bile and stool in which the nitric acid reagent requires 20 to 40 hours, while hydrogen peroxide acts almost as quickly as it does with pure bilirubin (Fig. 2). The hydrogen peroxide reagent has the further advantage of being relatively stable, although it should be kept in a dark bottle in the refrigerator, and should be made up fresh about once a month. Over this period of time there is no detectable diminution in its potency.

### *Method*

#### *Reagents—*

1. Oxidizing reagent. 0.4 cc. of 30 per cent hydrogen peroxide (superoxol) and 2 cc. of concentrated HCl in 100 cc. of 94 per cent ethyl alcohol.

2. 94 per cent ethyl alcohol.

*Bile Determinations*—9.5 cc. of ethyl alcohol and 10 cc. of oxidizing reagent are added to 0.5 cc. of bile. After 1 hour the solution is filtered through a No. 42 Whatman filter paper into a colorimeter tube and read in the colorimeter with Filter 660, with a tube containing 10 cc. of alcohol as a blank. If the galvanometer reading is less than 10, the solution should be diluted accurately with alcohol and read again.

*Meconium or Stool Determinations*—The 24 hour fresh specimen of meconium or infant's stool is transferred to a beaker, diluted with from 200 to 500 cc. of distilled water (depending upon the size of the specimen and the amount of bilirubin expected), and stirred with a mechanical stirrer for 3 to 5 hours until a homogeneous emulsion results. The essence of the procedure consists in obtaining an extremely fine suspension of stool particles. After measuring the total volume, a 10 cc. aliquot (meas-

ured with a 10 cc. graduated cylinder) is transferred to a 50 cc. centrifuge tube to which are added 15 cc. of alcohol and 25 cc. of oxidizing reagent. The solution is stirred vigorously, allowed to stand for 1 hour, and centrifuged. The supernatant liquid is poured off and allowed to stand until the second extraction has been carried out. The precipitate is drained by standing the tube upside down on filter paper for a few minutes, a further 25 cc. of alcohol and 25 cc. of oxidizing reagent are added, and the solution is stirred vigorously, allowed to stand for 1 hour, and filtered into a colorimeter tube. A portion of the first supernatant liquid is filtered into a second tube, and both tubes are read in the colorimeter with Filter 660, with alcohol as a blank.

The supernatant liquid of the first extraction, especially of meconium, may appear green rather than blue, owing to the presence of extraneous brown pigments, but it has already been pointed out that Filter 660 is unaffected by these pigments, and measures the blue color only.

A double extraction of meconium and stool specimens has been found necessary, since in our experience the first extraction only recovers about 80 per cent of the total bilirubin.

*Calculation*—The amount of bilirubin in mg. contained in the original specimen is obtained from the formula

$$\frac{2 - \log G}{K_1} \times \frac{V}{A} \times S$$

where  $G$  is the galvanometer reading,  $K_1$  is the calibration constant,  $V$  is the volume of colored solution,  $A$  is the aliquot of original specimen used, and  $S$  is the total volume of the original specimen. The calibration constant, determined with pure Hoffmann-La Roche bilirubin, has been found to be 78.0.

Thus for bile, if the 24 hour volume were  $S$  cc., the total amount of bilirubin would be

$$\frac{2 - \log G}{78.0} \times \frac{20}{0.5} \times S \text{ mg.}$$

And for meconium or stool, if the 24 hour *emulsion* were  $S$  cc., the total amount of bilirubin from the first extraction would be

$$\frac{2 - \log G_1}{78.0} \times \frac{50}{10} \times S \text{ mg.}$$

and from the second extraction

$$\frac{2 - \log G_2}{78.0} \times \frac{50}{10} \times S \text{ mg.}$$

Following the usual convention of writing  $(2 - \log G)$  as  $L$ , and adding together the yields obtained from the first and second extractions, we obtain for the total bilirubin

$$\frac{1}{78} \times \frac{50}{10} \times S \times (L_1 + L_2) \text{ mg.}$$

### Results

With this technique measurements may be made on solutions containing as little as 0.5 microgram of bilirubin per cc.

TABLE I  
*Recovery of Bilirubin*

The figures refer to micrograms of bilirubin per 10 cc. of colored solution.

Material	Bilirubin determined in sample	Bilirubin added	Total bilirubin calculated	Total bilirubin determined	Per cent recovery
Bile A	2.99	1.72	4.71	4.70	99.7
	2.99	2.58	5.57	5.54	99.2
	2.99	3.44	6.43	6.43	100.0
" B	2.80	1.37	4.17	4.20	100.8
	2.80	1.82	4.62	4.57	99.0
	2.80	2.74	5.54	5.40	97.5
	2.80	3.64	6.44	6.28	97.7
	2.80	4.54	7.34	7.18	97.8
Stool	2.55	2.58	5.13	5.00	97.5
	2.55	1.72	4.27	4.20	98.5
	2.55	3.44	5.99	5.73	95.7
	1.27	1.72	2.99	3.10	103.7
	5.30	1.72	7.02	7.00	99.7
Meconium	2.36	1.72	4.08	4.20	103.0
	2.36	2.58	4.94	4.98	100.8
	2.36	3.44	5.80	6.10	105.0
	1.18	1.72	2.90	2.82	97.3
	5.72	1.72	7.44	7.70	103.7

Table I shows that bilirubin may be recovered from bile with a maximum error of  $\pm 2$  per cent, and from stool or meconium with a maximum error of  $\pm 5$  per cent.

## SUMMARY

1. A spectrophotometric study has been made of the colors produced by the oxidation of bilirubin.

2. A comparison has been made of the behavior of three oxidizing reagents now in use for the determination of bilirubin in bile.

3. On the basis of the above studies, a simple and accurate method has been described for the determination of bilirubin in bile and meconium with the photoelectric colorimeter.

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# SYNTHESIS OF $\alpha$ -AMINO- $\beta$ -HYDROXY-*n*-BUTYRIC ACIDS

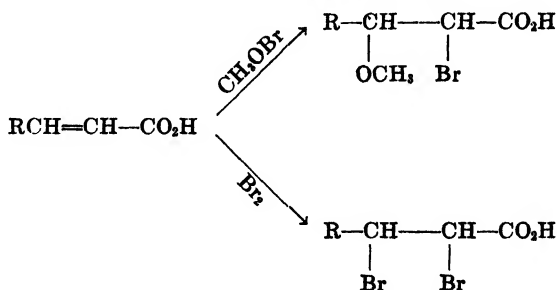
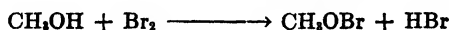
## V. PREPARATION OF *dl*-ALLOTHREONINE, WITH NOTES CONCERNING THE ADDITION OF METHYL HYPOBROMITE TO UNSATURATED ACIDS\*

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(Received for publication, November 6, 1937)

We have examined several methods of preparing  $\alpha$ -bromo- $\beta$ -methoxy acids in the hope of finding a reaction which would yield only one of the two racemic forms possible in the case of many such acids. Conant and Jackson (1) reported that cinnamic acid when treated with methyl alcohol and bromine yielded  $\alpha$ -bromo- $\beta$ -methoxy- $\beta$ -phenylpropionic acid together with dibromocinnamic acid. Only one form of the bromomethoxy acid was isolated. Therefore a study of the reaction of crotonic acid with methyl alcohol and bromine was undertaken. The reactions involved are shown in the accompanying equations.



\* The experimental data in this paper are taken from the thesis submitted by Harold D. West in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate School of the University of Illinois. Experimental work on the *cis*- and *trans*-cinnamic acids was carried out by Guy S. Krummel.

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If R in these reactions is aromatic, the addition of bromine is relatively slow and a fair yield of the bromomethoxy acid is obtained. However, in the case of crotonic acid the major product was found to be the dibromo acid. In order to make the reaction practical, therefore, it was necessary to decrease the amount of bromine added. Silver nitrate (2) and sodium acetate (3) have been used to shift the methyl alcohol-bromine equilibrium in favor of methyl hypobromite. The effect of these reagents on the reaction of crotonic acid with methyl alcohol and bromine was therefore studied. Sodium acetate increased the addition of methyl hypobromite only slightly. In the presence of silver nitrate, however, an excellent yield of  $\alpha$ -bromo- $\beta$ -methoxy-*n*-butyric acid was obtained. Only one racemic form could be isolated from the reaction mixture. Unfortunately it was not the isomer yielding *dl*-threonine. However, the reaction does furnish a simple method of obtaining the intermediate bromo acid used in the synthesis of *dl*-allothreonine.

In order to determine whether the *cis* and *trans* isomers of an unsaturated acid yield different forms of the bromomethoxy acid the reaction of cinnamic acid (*trans*) and allocinnamic acid (*cis*) with methyl alcohol and bromine was studied. In both cases, the use of silver nitrate increased the yield considerably. In agreement with the results of Conant and Jackson (1) only one  $\alpha$ -bromo- $\beta$ -methoxy- $\beta$ -phenylpropionic acid (m.p. 183–184°) was obtained from cinnamic acid. Allocinnamic acid yielded mainly the second form (m.p. 139–140°). However, a small amount of the higher melting isomer was also isolated from the reaction mixture. Since allocinnamic acid is readily changed to cinnamic acid, it seems probable that the higher melting isomer came, not directly from allocinnamic acid, but rather from a small amount of the *trans* isomer produced under the conditions of the reaction. Since *cis* acids are generally changed into the *trans* form by the action of bromine and also of hydrogen ion, it seems likely that a mixture of bromomethoxy acids will usually be obtained from a *cis* acid by this reaction. On the other hand, all of the *trans* acids studied yielded a single bromomethoxy acid. The second form was not present in the reaction mixture in an amount sufficient to be detected.

Jackson and Pasiut (4) prepared an iodomethoxybutyric acid

of undetermined structure by the action of iodine monochloride on a solution of crotonic acid in methyl alcohol. Since it seemed very probable that the substance was an  $\alpha$ -iodo- $\beta$ -methoxybutyric acid, we prepared a small amount of the material and found that it yielded *dl*-allothreonine on amination and demethylation. Therefore, the substance is an  $\alpha$ -iodo- $\beta$ -methoxybutyric acid having the same optical configuration as the  $\alpha$ -bromo- $\beta$ -methoxybutyric acid obtained by the addition of methyl hypobromite to crotonic acid.

#### EXPERIMENTAL

##### *General Method*

170 gm. (1 mole) of finely powdered silver nitrate and 1000 cc. of methyl alcohol were placed in a 3 liter 3-necked flask fitted with two dropping funnels and an efficient stirrer. The flask was cooled in an ice bath and the contents were stirred for 5 to 10 minutes. Then 160 gm. (1 mole) of bromine and a solution of 1 mole of the unsaturated acid in the necessary amount of methyl alcohol were added slowly through the dropping funnels. The contents of the flask were held at 5–15° and vigorously stirred during the addition (1.5 to 2 hours) and for 2 hours after the reagents were added. The flask was then allowed to come slowly to room temperature by remaining in the ice bath overnight. The silver bromide was removed by filtration and any excess bromine in the filtrate was destroyed with sodium bisulfite. The solution was made just alkaline to phenolphthalein with 2 N sodium hydroxide and concentrated to a small volume under reduced pressure. The concentrated solution was filtered, cooled, and extracted once with ether. 6 N sulfuric acid was then added slowly with cooling and shaking until no more bromo acid precipitated. The mixture was then extracted with ether (four extractions were made in the case of crotonic acid, only one in the case of cinnamic acid). The ether solution was washed twice with small volumes of cold water and dried over anhydrous sodium sulfate. The ether was distilled, leaving the crude bromo acid which was purified either by recrystallization or fractional distillation under reduced pressure.

*$\alpha$ -Bromo- $\beta$ -Methoxy-*n*-Butyric Acid*—The crude bromo acid was distilled under reduced pressure. After a small amount of

low boiling material was removed, the remainder distilled in one fraction at 126–130° at 8 mm. The distillate crystallized in the receiver to a solid melting at 58–61°. The yield from 1 mole of crotonic acid was 130 to 135 gm. (66 to 68.5 per cent of the theoretical amount). Recrystallization of the material from a benzene-petroleum ether mixture raises the melting point to 62–63°.

*dl-Allothreonine*—The crude undistilled bromo acid was aminated and demethylated as previously described (5). The yield of pure *dl*-allothreonine from 1 mole of crotonic acid was 35 to 43 gm. (25 to 33 per cent of the theoretical amount).

*α-Bromo-β-Methoxy-β-Phenylpropionic Acid. From Cinnamic Acid*—The crude bromo acid was dissolved with warming in 10 volumes of 10 per cent sodium carbonate and the solution was cooled overnight in the ice box. The precipitate (sodium salt of the bromo acid melting at 183–184°) was removed by filtration and dissolved in 20 volumes of water. The solution was acidified with dilute hydrochloric acid and the precipitate was extracted with ether. The ether solution was dried and the ether removed. The crystalline solid left melted at 176–181° and therefore was the higher melting bromo acid in nearly pure state. The original filtrate was acidified with dilute hydrochloric acid and extracted with ether. The ether was dried and distilled, leaving a small amount of a viscous gum. The only crystalline material isolated from the gum was a further small amount of the bromo acid melting at 183–184°. The yield from 1 mole of cinnamic acid was 180 to 195 gm. (69.5 to 75 per cent of the theoretical amount).

*From Allocinnamic Acid*—The procedure described for cinnamic acid was followed in this case also. 14.8 gm. of allocinnamic acid gave 3 gm. of the bromo acid melting at 183–184°, isolated as the sodium salt. When the soluble sodium salt fraction was worked up, 15 gm. of bromo acid were obtained melting at 132–137°. This material still contained a small amount of the higher melting isomer which was removed only after several recrystallizations from benzene accompanied by considerable loss of material.

#### SUMMARY

1. Methyl hypobromite reacts with an  $\alpha, \beta$ -unsaturated acid, yielding an  $\alpha$ -bromo- $\beta$ -methoxy acid. Good yields are obtained if the reaction is carried out in the presence of silver nitrate.

2. The bromomethoxy acid obtained from crotonic acid yields *dl*-allothreonine on amination and demethylation.

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## SYNTHESIS OF $\alpha$ -AMINO- $\beta$ -HYDROXY-*n*-BUTYRIC ACIDS

### VI. PREPARATION OF *d*- AND *l*-ALLOTHREONINE AND NUTRITIVE VALUE OF THE FOUR ISOMERS\*

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(Received for publication, November 6, 1937)

In Paper IV of this series (1) the synthesis of *d*(-)- and *l*(+)-threonine was described. We have since prepared *d*- and *l*-allothreonine by similar methods, thus making available the four optically active  $\alpha$ -amino- $\beta$ -hydroxy-*n*-butyric acids. It, therefore, seemed desirable to study the behavior of the four isomers in growth experiments, although the inability of *dl*-allothreonine to support growth of rats (2) rendered it very unlikely that either of the optically active forms would do so. The present paper reports the preparation of *d*- and *l*-allothreonine and the results of a study of the nutritive value of the four optically active  $\alpha$ -amino- $\beta$ -hydroxy-*n*-butyric acids.

#### EXPERIMENTAL

*Preparation of d- and l-Allothreonine*—*dl*-O-Methylallothreonine was synthesized by the method of West, Krummel, and Carter (3) and was converted into the formyl derivative in the manner previously described (2). 153.4 gm. of formyl-*dl*-O-methylallothreonine were dissolved in 1540 cc. of boiling absolute alcohol and added to a solution of 372 gm. of brucine in 1200 cc. of boiling absolute alcohol. The mixture was placed in an ice box overnight. The precipitate was removed by filtration, washed with cold alcohol, and dried. This material was recrystallized three times from

\* The experimental data in this paper are taken from a thesis submitted by Harold D. West in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate School of the University of Illinois.

† Fellow of the General Education Board, New York.

TABLE I  
Constants and Analyses

Substance	M.p.	$[\alpha]_D^{25}$	Formula	N analyses	
				Calculated	Found
	°C.	degrees		per cent	per cent
Brucine formyl- <i>l</i> -O-methylallothreonine.....	132-136	-22.5	$C_{21}H_{27}O_5N_3$	7.57	7.56
Brucine formyl- <i>d</i> -O-methylallothreonine.....	186-188	-19.9	"	7.57	7.63
<i>l</i> -Allothreonine.....	269-272	-9.11	$C_4H_9O_3N$	11.76	11.70
<i>d</i> -Allothreonine.....	268-272	+9.60	"	11.76	11.84
N-Benzoyl- <i>l</i> -allothreonine....	127-128	-17.0	$C_{11}H_{13}O_4N$	6.28	6.39
N-Benzoyl- <i>d</i> -allothreonine....	128-129	+17.1	"	6.28	6.31

TABLE II  
Composition (in Gm.) of the Diets\*

	Diet I	Diet II	Diet III	Diet IV	Diet V
Amino acid Mixture XX†.....	20.5	20.5	20.5	20.5	20.5
Dextrin.....	27.5	26.8	26.8	26.8	26.8
Sucrose.....	15.0	15.0	15.0	15.0	15.0
Salt mixture‡.....	4.0	4.0	4.0	4.0	4.0
Agar-agar.....	2.0	2.0	2.0	2.0	2.0
Lard.....	26.0	26.0	26.0	26.0	26.0
Cod liver oil.....	5.0	5.0	5.0	5.0	5.0
<i>d</i> (-)-Threonine.....	0.0	0.7	0.0	0.0	0.0
<i>l</i> (+)-Threonine.....	0.0	0.0	0.7	0.0	0.0
<i>l</i> -Allothreonine.....	0.0	0.0	0.0	0.7	0.0
<i>d</i> -Allothreonine.....	0.0	0.0	0.0	0.0	0.7
	100.0	100.0	100.0	100.0	100.0

\* Each diet contained 12.3 per cent of "effective" amino acid exclusive of the supplement. The vitamin B factors were supplied in the form of two pills daily, each containing 50 mg. of tikitiki extract and 75 mg. of milk concentrate.

† Rose and coworkers (7).

‡ Osborne and Mendel (8).

absolute alcohol (9 cc. per gm.) which gave a pure salt (brucine formyl-*l*-O-methylallothreonine). The original mother liquor was concentrated to one-half its volume and allowed to stand over-

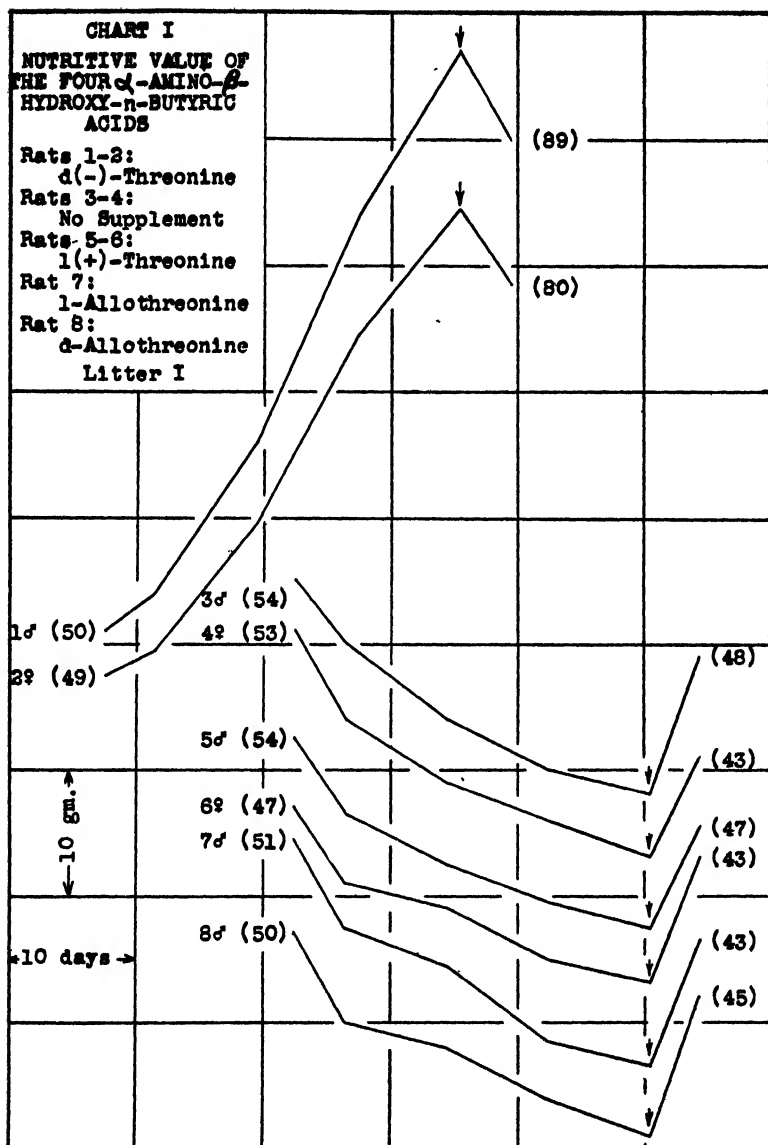


CHART I. The numbers in parentheses denote the initial and final weights of the rats. At the arrows the following dietary changes were made: Rats 1 and 2 were deprived of  $d(-)$ -threonine and Rats 3 to 8 were given  $d(-)$ -threonine.



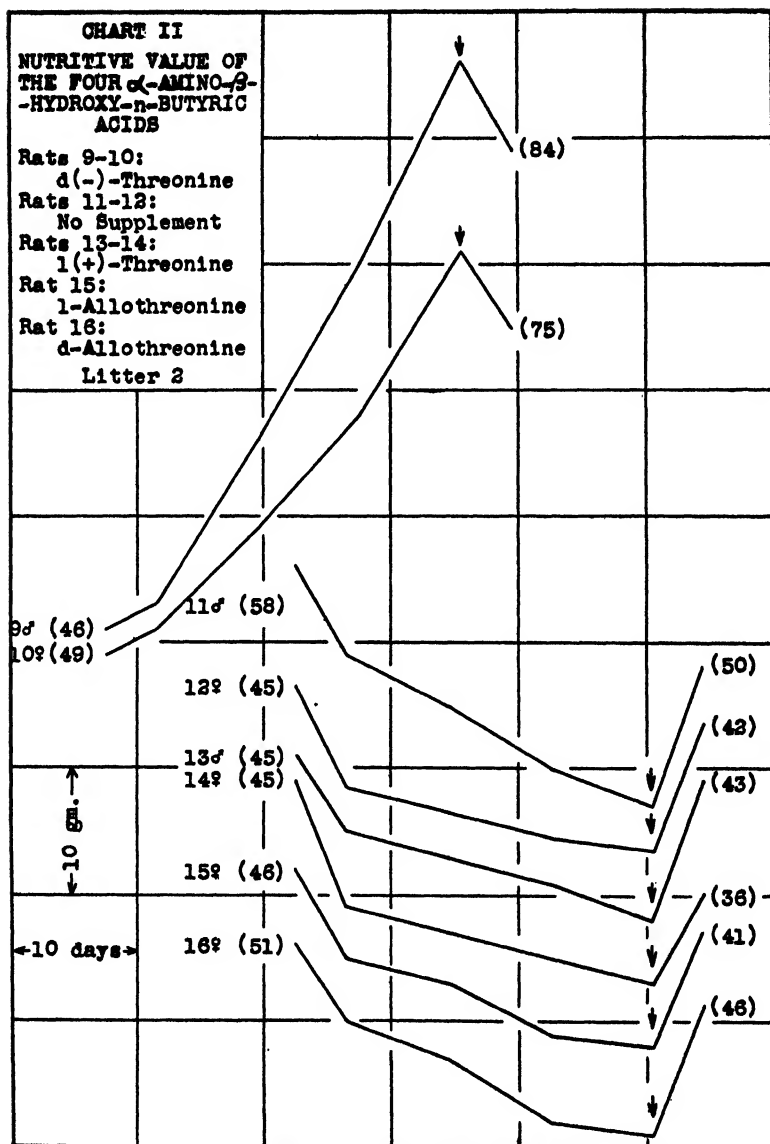


CHART II. The numbers in parentheses denote the initial and final weights of the rats. At the arrows the following dietary changes were made: Rats 9 and 10 were deprived of d(-)-threonine and Rats 11 to 16 were given d(-)-threonine.

night in the ice box. The precipitate was removed by filtration, dried, and recrystallized three times from absolute alcohol,

TABLE III  
*Changes in Body Weight and Food Intakes of Rats*

Litter No.	Rat No. and sex	Duration of experiment	Total weight change	Total food intake	Supplement
		<i>days</i>	<i>gm.</i>	<i>gm.</i>	
1	1 ♂	28	+46	112	<i>d</i> (-)-Threonine
		4	-7	13	None
	2 ♀	28	+37	102	<i>d</i> (-)-Threonine
		4	-6	16	None
	3 ♂	28	-17	43	"
		4	+11	13	<i>d</i> (-)-Threonine
	4 ♀	28	-18	47	None
		4	+8	11	<i>d</i> (-)-Threonine
	5 ♂	28	-15	46	<i>l</i> (+)-Threonine
		4	+8	11	<i>d</i> (-)-Threonine
	6 ♀	28	-14	40	<i>l</i> (+)-Threonine
		4	+10	13	<i>d</i> (-)-Threonine
	7 ♂	28	-18	40	<i>l</i> -Allothreonine
		4	+10	12	<i>d</i> (-)-Threonine
	8 ♂	28	-16	40	<i>d</i> -Allothreonine
		4	+11	13	<i>d</i> (-)-Threonine
2	9 ♂	28	+45	113	"
		4	-7	12	None
	10 ♀	28	+32	99	<i>d</i> (-)-Threonine
		4	-6	11	None
	11 ♂	28	-19	47	"
		4	+11	14	<i>d</i> (-)-Threonine
	12 ♀	28	-13	42	None
		4	+10	12	<i>d</i> (-)-Threonine
	13 ♂	28	-13	39	<i>l</i> (+)-Threonine
		4	+11	13	<i>d</i> (-)-Threonine
	14 ♀	28	-16	36	<i>l</i> (+)-Threonine
		4	+7	10	<i>d</i> (-)-Threonine
	15 ♀	28	-14	43	<i>l</i> -Allothreonine
		4	+9	12	<i>d</i> (-)-Threonine
	16 ♀	28	-15	44	<i>d</i> -Allothreonine
		4	+10	13	<i>d</i> (-)-Threonine

yielding practically pure brucine formyl-*d*-O-methylallothreonine. The brucine salts were converted into *l*- and *d*-allothreonine by

the method used for preparing *d*(-)- and *l*(+)- threonine (1). The intermediate products were not purified. N-Benzoyl derivatives of *d*- and *l*-allothreonine were prepared by the method of Sørensen and Andersen (4). The filtrates from the benzoic acid were concentrated and cooled overnight in the ice box. The crude benzoyl derivatives precipitated and were purified by recrystallization from ethyl acetate.

The constants and analyses of the above substances are summarized in Table I.

*Growth Experiments*—The feeding experiments were carried out by the method of Rose and coworkers (5, 6). The composition of the diets is shown in Table II. Amino acid Mixture XX, which is being used in this laboratory at the present time, was devised by Rose and coworkers, who will report its composition later. Diet I was an otherwise adequate threonine-free mixture. Diets II, III, IV, and V contained *d*(-)-threonine, *l*(+)-threonine, *l*-allothreonine, and *d*-allothreonine respectively. At the end of the usual 28 day feeding period the rats receiving Diet II were changed to Diet I and the rats receiving Diets I, III, IV, and V were shifted to Diet II for an additional 4 day period. The results are shown in Charts I and II. The changes in body weight and the food consumption of the rats are shown in Table III.

As we had expected, only the naturally occurring *d*(-)-threonine is utilized by the rat for growth purposes. It cannot be replaced by any other of the isomers. This is a remarkable example of the stereochemical specificity of animal tissue. One form of allothreonine has the same configuration of the  $\alpha$ -carbon atom as *d*(-)- threonine. The other form has the same configuration of the  $\beta$ -carbon atom as *d*(-)- threonine. Yet neither of these isomers can be utilized by the rat for growth purposes in lieu of the natural form.

In concluding our work in this field, we wish to express our appreciation to Professor W. C. Rose for allowing us the privilege of assisting in this interesting problem.

#### SUMMARY

1. *d*- and *l*-allothreonine have been synthesized.
2. Growth experiments have demonstrated that *d*(-)-threonine is the only one of the four  $\alpha$ -amino- $\beta$ -hydroxy-*n*-butyric

acids which will support the growth of rats on an otherwise adequate diet.

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## THE INFLUENCE OF AGE, SEX, AND OVARIAN ACTIVITY ON THE BLOOD LIPIDS OF THE DOMESTIC FOWL\*

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Although several reports on the blood lipids of the bird have appeared, they have been fragmentary in nature and in most cases have dealt with a single lipid constituent (1-4). Warner and Edmond (1), in noting that the blood of the laying bird contained more fat than that of the non-laying, found no correlation between blood fat and egg yield. A rise in fatty acids and lipid phosphorus in the laying bird was also reported by Lawrence and Riddle (2). These workers further stated that the plasma of the non-laying female contained more fatty acids than the plasma of the male bird, but this finding was not confirmed in the more extensive work of Warner and Edmond (1). Kaishio (3) found no difference in the cholesterol content of the blood of non-laying female and male birds.

The actively laying domestic fowl provides a good experimental animal for the study of a rapid fat metabolism. Its unique nature in this respect can be inferred from the fact that, in addition to its normal caloric expenditure, a 1000 gm. bird loses approximately 4 gm. of fat daily to maintain the not uncommon laying rate of one egg per day for more or less extended periods. In order to throw light on the mechanism involved, a series of studies has been undertaken on the lipid metabolism of this animal. In the present investigation all blood lipid constituents, namely total fatty acids, cholesterol (both free and esterified), and phos-

\* Aided by a grant from the Research Board of the University of California. The assistance given by the Works Progress Administration is gratefully acknowledged.

pholipids, have been followed from early age through puberty and up to maturity in both male and female birds.

#### EXPERIMENTAL

Single comb white Leghorns of the Poultry Division stock were used in this investigation. They were fed two types of diets, differing in fat and carbohydrate contents. The low fat diet had

TABLE I  
*Low Fat Diet*

	<i>per cent</i>
Ether-extracted sardine meal.....	15.0
Commercial casein.....	5.0
Ground wheat.....	20.0
Commercial corn-starch.....	40.4
Dehydrated alfalfa leaf meal.....	8.0
Rice bran concentrate*.....	3.0
Salts†.....	0.5
Ground oyster shell.....	0.5
Cotton pulp.....	6.5
Agar‡.....	1.0
Fish oil mixture§.....	0.01

\* The rice bran concentrate was kindly furnished by Vitab Products, Inc. This concentrate contained 55 international units of vitamin B<sub>1</sub> and 10 modified Bourquin-Sherman units of vitamin G (flavin) per cc. The same concentrate has also been shown to be a good source of both rat and chick antidermatitis factors (5).

† The salt mixture contained 1.0 per cent ferric citrate and 0.1 per cent copper sulfate.

‡ The agar was added as a warm solution and this, when dried, gave the diet a granular consistency.

§ This contained 20,000 international units of vitamin A and 12,333 international units of vitamin D per gm. It was kindly furnished by the F. E. Booth Company.

the composition shown in Table I. This ration contained approximately 18 per cent protein, 60 per cent carbohydrate, and less than 1 per cent fat. Calcium and phosphorus were present to the extent of 1.02 and 0.53 per cent respectively.

The high fat diet contained the constituents listed above, except that the starch and agar were replaced by 17.3 per cent Crisco and 24.1 per cent cotton pulp. This diet, containing approxi-

mately 18 per cent fat and 20 per cent carbohydrate, had approximately the same calorific value per unit of weight as the low fat diet.

Groups of chicks were placed on these two diets at the age of 4 weeks. Before this they had been maintained on the standardized chick mash employed in this laboratory. The chicks were kept in broiler batteries until they approached maturity, when they were transferred to individual laying batteries. Before the onset of laying, both diets were modified by the addition of 3.0 per cent bone ash and by increasing the oyster shell level from 0.5 to 4.0 per cent. An equivalent amount of cotton pulp was eliminated from the diet at this time. These changes increased the calcium and phosphorus respectively to 3.47 and 1.05 per cent, a level optimum for egg production, without causing alterations in the fat, carbohydrate, and protein percentages of the diets. Egg production records of each bird were kept.

Growth on these two diets was essentially normal, as judged by data obtained in this laboratory over a period of many years. No significant differences were observed in the growth rates on the high and low fat diets.

Food was supplied to the birds *ad libitum* except at those times when blood was taken. Blood was removed by heart puncture between 8 and 10 a.m.; before this the animal had been deprived of food for 16 hours.

Microoxidative procedures were employed for lipid determinations. These have been described elsewhere (6).

#### *Blood Lipids of Male Bird*

*Low Fat Diet*<sup>1</sup>—Blood lipid constituents were determined in seven birds fed a low fat diet at intervals between the ages of 71 and 276 days. Table II summarizes the mean values for this and subsequent groups of birds. The total lipid content of whole blood varied from 376 to 530 mg. per cent, while the minimum and maximum values for fatty acids were 267 and 425 mg. per cent, respectively. The values for total cholesterol obtained in these seven birds were 102 for the lowest and 147 for the highest;

<sup>1</sup> While the terms high and low fat are used to describe the diets employed in this study, it should be noted that the carbohydrate content of the two diets was not the same.



TABLE II  
*Mean Values and Standard Errors of Whole Blood Lipids of Male and Female Birds\**  
 The values are expressed in mg. per 100 cc. of whole blood.

	Fat of diet	No. of analyses	Cholesterol			Total fatty acids	Phospholipid	Total lipid	Residual fatty acids
			Total	Free	Ester				
Males	Low	12	114 ± 4	92 ± 4	22 ± 3	314 ± 13	299 ± 8	428 ± 13	99 ± 13
	High	12	131 ± 7	88 ± 3	42 ± 6	331 ± 18	304 ± 10	461 ± 22	97 ± 13
Immature females	Low	7	117 ± 8	83 ± 4	35 ± 6	329 ± 19	282 ± 11	446 ± 25	115 ± 16
	High	6	119 ± 8	80 ± 4	39 ± 7	361 ± 33	288 ± 12	480 ± 41	140 ± 23
Laying	Low	36	125 ± 5	109 ± 4	16 ± 3	1564 ± 146	642 ± 33	1689 ± 149	1123 ± 133
	High	32	136 ± 6	108 ± 5	29 ± 4	1209 ± 110	572 ± 30	1345 ± 115	795 ± 94

\* Values in Tables II to V were obtained in the postabsorptive state.

the free or uncombined cholesterol fluctuated from 68 to 106 mg. per cent, whereas the esterified portion varied from 6 to 43 mg. These variations in lipid values were in no way related to the age of the bird. Thus, while the mean value for total lipid was  $428 \pm 13$  mg. per cent,<sup>2</sup> for two birds of the same age, namely 215 days, 376 and 430 mg. per cent were found. Total cholesterol remained fairly constant, with a mean of  $114 \pm 4$  mg. per cent, while the free cholesterol fraction averaged  $92 \pm 4$  mg. per cent. The esterified cholesterol showed the greatest fluctuation of the lipid constituents studied with the low fat diet, but again the fluctuation was in no way related to the bird's age.

*High Fat Diet*—Nine birds were maintained on this diet; the mean blood lipid values obtained between the ages of 71 to 276 days are recorded in Table II. Maximum and minimum values for the various lipid constituents were as follows: total cholesterol, 187 and 103 mg. per cent; free cholesterol, 100 and 74 mg.; ester cholesterol, 90 and 15; phospholipids, 360 and 253; total fatty acids, 464 and 252; total lipid, 651 and 373. As in the case of the low fat diet, age played no part in the variations observed in the level of the blood lipid constituents of the male bird.

#### *Blood Lipids of Female Bird*

*Immature Bird*—The mean values obtained for the blood lipid constituents of twelve immature birds maintained on low and high fat diets are shown in Table II. Blood was removed from these animals at intervals between 71 and 135 days of age. The values found did not differ significantly from those observed in the males. The range of values for all immature female birds studied was as follows: total cholesterol, 92 to 155 mg. per 100 cc. of whole blood; free cholesterol, 72 to 106; ester cholesterol, 20 to 65; phospholipids, 225 to 328; total fatty acids, 283 to 494; total lipid, 389 to 649. As may be seen from Table II, statistically significant differences were not found between the high and low fat diets.

*Female Bird during Puberty*—For the purpose of these lipid studies, a bird is described as mature when the first egg is laid. Puberty has been arbitrarily defined as the interval between the

<sup>2</sup> Standard errors are used throughout.

onset of accelerated comb growth and maturity. The blood lipid values found in female birds during this stage are shown in Table III. A striking change was found in the lipid concentration in the blood of a number of these birds. In three of the five birds

TABLE III  
*Whole Blood Lipids of Pubescent Females*

The values are expressed in mg. per 100 cc. of whole blood.

Bird No.	Age	Weight	Days before maturity	Cholesterol			Total fatty acids	Phospho-lipid	Total lipid*	Resid-ual fatty acids
				Total	Free	Ester				
Low fat diet										
	<i>days</i>	<i>kg.</i>								
16	157	1.3	25	101	76	25	358	272	459	158
28	157	1.5	10	118	103	15	799	470	917	473
24	157	1.5	3	198	184	14	2863		3061	
09	171	1.6	(2 ova)†	210	176	34	2828	905	3038	2197
22	217	1.4	(3 " )†	189	168	21	2592	967	2781	1929
High fat diet										
55	171	1.7	(4 ova)†	127	103	24	1249	619	1376	817
62	157	1.3	35	110	72	38	285	252	395	88
65	162	1.5	14	126	90	36	328	332	454	79
75	162	1.4	21	93	73	20	403	353	496	152
75	174	1.6	9	238	189	49	3320	1131	3558	2526
78	150	1.3	26	114	80	34	300	242	414	13
72	217	1.4	(3 ova)†	143	127	16	1883	660	2026	1429

\* Total lipid represents the sum of total fatty acids and total cholesterol (6).

† Fatty acids other than those in combination with cholesterol and phospholipid. These fatty acids are derived chiefly from neutral fat (triglycerides).

‡ These birds were killed before maturity was attained. The figures in parentheses refer to the number of actively growing ova greater than 0.5 cm. in diameter that were found when blood was taken.

maintained on the low fat diet the lipids had attained enormous concentrations in the blood, the highest being found in Bird 22, in which total lipids had risen to 2781 mg. per cent. High lipid values in the blood were also observed in three of the birds that received the high fat diet. All lipid constituents did not

TABLE IV

*Whole Blood Lipids of Laying Birds on Low Fat Diet*

The values are expressed in mg. per 100 cc. of whole blood.

Bird No.	Age	Weight	Egg record		Cholesterol			Total fatty acids	Phospho-lipid	Total lipid	Residual fatty acids
			Pro-duction to date*	Inten-sity of pro-duction†	Total	Free	Ester				
	<i>days</i>	<i>kg.</i>									
02	162	1.3	2	0.70	129	128	1	1608	541	1737	1245
(160)‡	174	1.3	10	0.64	89	92	0	996	532	1085	637
	189	1.3	20	0.71	154	129	25	2999	860	3153	2404
	195	1.4	23	0.64	89	86	3	975	544	1064	597
	202	1.3	28	0.71	155	148	7	1907	752	2062	1398
	269	1.3	76	0.71	73	67	6	506	404	579	231
	283	1.3	86	0.71	122	116	7	1362	642	1484	927
	299	1.3	93	0.57	102	99	3	1323	561	1425	945
04	162	1.5	2	0.90	129	116	13	1895	780	2024	1363
(160)	174	1.5	10	0.64	107	93	14	1262	569	1369	871
	189	1.5	20	0.79	95	72	23	548	553	643	161
	195	1.5	24	0.71	108	92	16	1205	510	1313	852
	210	1.5	34	0.64	102	102	0	985	473	1087	668
	276	1.7	58	0.50	91	72	19	760	524	851	395
08	210	1.9	17	0.57	126	110	16	1150	575	1276	754
(201)	220	2.0	25	0.57	164	109	55	1126	525	1290	734
11	174	1.7	1	0.45	106	105	1	1278	507	1384	938
(171)	185	1.7	6	0.64	138	87	51	787	471	925	434
	203	1.7	19	0.71	123	79	44	945	550	1068	544
	205	1.7	21	0.50	99	86	13	829	473	928	503
	210	1.7	24	0.64	124	124	0	1503	670	1627	1054
	218	1.7	29	0.57	93	98	0	1031	493	1124	701
	269	1.9	51	0.43	112	94	18	4607	515	4719	4249
	283	2.0	57	0.50	157	145	12	2329	909	2486	1711
26	162	1.3	1	0.60	124	108	16	1481	615	1605	1057
(160)	219	1.3	10	0	95	97	0	1003	351	1098	768
28	174	1.7	4	0.57	141	134	7	2502	837	2643	1936
(167)	192	1.7	17	0.86	127	119	8	1905	652	2032	1462
	203	1.7	24	0.64	162	149	13	1842	846	2004	1266
	205	1.7	26	0.64	119	93	26	911	653	1030	455
	211	1.7	30	0.71	167	137	30	2173	891	2340	1554
	276	1.8	77	0.57	155	107	48	1264	603	1419	825
	283	1.9	80	0.57	161	122	39	2262	860	2423	1657
	299	1.8	90	0.71	215	206	9	3914	1485	4129	2912

\* Refers to the total number of eggs laid by the time of removal of the blood sample.

† Refers to the number of eggs per day during the period of active laying limited by 7 days preceding and 7 days after the blood was examined.

‡ Figures in parentheses refer to the age of the bird in days when first egg was laid.

share equally when total lipid increases occurred. In comparison with the immature animals shown in Table II, a rise may occur in total cholesterol during puberty. The two constituents, however, that suffer the greatest change when total lipids rise are neutral fat and phospholipids. Although phospholipid values as high as 905 and 967 mg. per cent were found, the rise in this constituent did not always keep pace with the rise in total fatty acids. Thus in Bird 75 the phospholipid content of the blood was 451 mg. at the same time that total fatty acids attained a value of 3320 mg. per cent.

Although sufficient data are not available for delimiting sharply the time of onset of blood lipid rises during puberty, it is nevertheless interesting to note that a high fat content of the blood was present when the interval before laying was 10 days or less. Four birds in puberty were sacrificed for examination of ovarian activity: rapidly growing ova were found, indicating that egg laying would begin within 10 days; the concentration of lipids in the blood of these birds also showed considerable elevation above the normal.

*Laying Bird*—The blood lipids of laying birds that received low and high fat diets are shown in Tables IV and V respectively. In twelve birds several determinations were made over periods that varied from 2 to 137 days. The high total lipid concentration of the blood, first observed in some birds during puberty, was found in all animals actively engaged in egg laying. Bird 65 showed a total lipid value of 551 mg. per cent at an interval of 6 days after the onset of laying, but it should be noted that values of 1033, 1077, and 1467 mg. per cent for total lipids were found in the same bird at other times during the laying period. The variations that occur in the lipid level of a laying bird are indeed striking. The rapidity with which the animal in this condition can effect changes in the fat concentration of the blood is well shown by Bird 28. Total lipids in the blood of this animal fell from 2008 to 1030 mg. per cent in the short time of 48 hours. 131 days after the onset of laying, total lipids in this animal reached the enormous figure of 4129 mg. per cent.

Although two values for total cholesterol well over 200 mg. per cent were found in Birds 75 and 28, this lipid constituent showed the least change during the laying period. Significant

TABLE V

*Whole Blood Lipids of Laying Birds on High Fat Diet*

The values are expressed in mg. per 100 cc. of whole blood.

Bird No.	Age	Weight	Egg record		Cholesterol			Total fatty acids	Phospho-lipid	Total lipid	Residual fatty acids
			Pro-duction to date*	Inten-sity of pro-duction*	Total	Free	Ester				
	<i>days</i>	<i>kg.</i>									
52	162	1.3	1	0.60	141	136	5	1918	795	2059	1382
(160)*	174	1.3	8	0.50	79	77	2	712	427	791	433
61	204	1.7	8	0.86	110	91	19	658	401	768	375
(195)	211	1.7	14	0.86	93	91	2	635	391	728	372
	269	1.8	58	0.57	128	99	29	824	473	952	486
	283	1.7	65	0.57	118	100	18	884	503	1002	534
62	192	1.5	1	0.75	165	110	55	1701	741	1866	1164
(192)	202	1.4	7	0.71	99	96	3	823	377	922	568
	205	1.4	10	0.64	128	73	55	549	391	677	247
	269	1.5	54	0.64	168	111	57	1566	697	1734	1057
	283	1.5	63	0.64	115	86	29	1227	561	1342	830
65	185	1.7	5	0.50	110	88	22	923	530	1033	552
(176)	192	1.7	8	0.57	97	93	4	454	441	551	156
	211	1.7	20	0.57	109	93	16	968	558	1077	582
	220	1.8	25	0.71	129	105	24	1338	653	1467	883
75	185	1.6	1	0.50	242	160	82	2403	850	2645	1774
(183)	189	1.7	3	0.57	158	124	34	1892	837	2050	1306
	192	1.7	4	0.57	166	155	11	2088	783	2254	1555
	205	1.7	11	0.57	173	171	2	2424	844	2597	1857
	210	1.7	14	0.57	161	134	27	2100	840	2261	1517
	218	1.7	19	0.50	195	190	5	1489		1684	
	276	2.0	35	0.43	148	117	31	1656	630	1804	1211
	283	1.9	38	0.57	182	117	65	1505	646	1687	1025
78	185	1.4	2	0	134	73	61	307	291	441	68
(176)	192	1.5	2	0	123	80	43	486	369	609	207
	210	1.5	6	0.64	128	75	53	709	450	837	369
	218	1.5	12	0.57	112	76	36	695	473	807	352
	269	1.7	34	0.14	153	112	41	1110	583	1263	689
	283	1.8	36	0.29	124	84	40	751	515	875	377

\* See explanations below Table IV.

rises in free cholesterol occurred during laying, but values comparable with those found in the immature state were also noted at times in laying birds that were studied for long periods. A

single determination on Bird 75 (high fat diet) while in the immature state showed a free cholesterol concentration of 75 mg. per 100 cc. of the whole blood. Eight bloods obtained over a period of 98 days during the time this bird was engaged in egg laying showed values for free cholesterol ranging from 124 to 190 mg. per cent. A slight rise in free cholesterol above the single value obtained in the immature state was noted in (laying) Bird 61. In Bird 62, maintained on a high fat diet, three of the four values obtained during the laying state were above the level found in immaturity. Essentially similar changes occurred in the free cholesterol of birds that had received the low fat diet (Table IV). Blood was removed on eight different occasions from Bird 28, and in all samples the free cholesterol level was above that observed during the immature state. Considerable fluctuations in free cholesterol, however, occurred in Birds 02, 04, and 11. The birds on the low fat diet had an average free cholesterol of  $109 \pm 4$  mg. per cent, while those on the high fat diet had a mean of  $108 \pm 5$  mg. Both these values are significantly higher than the corresponding means found for immature birds.

The most striking changes during laying occurred in neutral fat and in phospholipids. But the response of these constituents was by no means uniform even in birds maintained on the same diet. The mean values for phospholipids in laying birds maintained on low and high fat diets were  $642 \pm 33$  and  $572 \pm 30$  mg. per cent, respectively, as compared with  $282 \pm 11$  and  $288 \pm 12$  mg. for non-laying birds. The highest value was found in Bird 28, in which it rose to 1485 mg. per cent. Mean values of  $1564 \pm 146$  and  $1209 \pm 110$  mg. per cent were found for total fatty acids in laying birds maintained on low and high fat diets respectively. In spite of the large standard errors, these values are significantly higher than corresponding mean values in immature birds.

#### DISCUSSION

The lipid concentrations in the whole blood of the immature female and male bird differ but slightly from the range of values found in other species such as man (7) and dog (8). This is clearly shown in the total fatty acid fraction. In the non-laying bird total fatty acids were present to the extent of  $330 \pm 11$  mg.

per cent,<sup>3</sup> whereas in the whole blood of the dog and children  $362 \pm 6$  and  $304 \pm 6$  mg. per cent have been found. Phospholipid values in the three species also corresponded closely: man, bird, and dog contained respectively  $236 \pm 6$ ,  $295 \pm 5$ , and  $324 \pm 7$  mg. per 100 cc. of whole blood. The mean values for total cholesterol in the immature female and male bird ( $121 \pm 3$  mg. per cent) were somewhat lower when compared with those in man ( $170 \pm 5$ ) and dog ( $156 \pm 3$ ), but here again no striking variations were found in the three types of animals.

In the absence of disease, age has no significant influence on the lipid level of human plasma (9). This apparently is also true in the male bird, which in this investigation was followed on high and low fat diets from ages of 71 to 276 days. On both diets no significant differences were found between the earliest and latest lipid concentrations found in whole blood. In the female bird, however, a most pronounced increase characterizes the lipid concentration of the blood while the animal is actively engaged in egg laying. This rise is confined largely to neutral fat, though free cholesterol and phospholipids also show statistically significant increases. Actual oviposition is not the stimulus for the rise in the blood fat, for significant increases may appear in birds during puberty. The rise in the lipid content of the blood first appears in late puberty, close to the time of the appearance of the first egg. Although the effects of diet will be dealt with below, it should be noted at this point that formation of fat from non-fat precursors can be a prominent feature in the laying bird, for enormous concentrations of neutral fat appeared in the blood of laying birds maintained on a low fat diet. During an interval of 7 days an actively laying bird (five eggs per week) excretes in the yolk approximately 20 gm. of total fatty acids, 1.4 gm. of cholesterol, and 6 gm. of phospholipids at the same time that it ingests approximately 7 gm. of total fatty acids and traces of cholesterol and phospholipids.

*Relation of Amount of Fat in Diet<sup>4</sup> to Level of Blood Lipids—Two*

<sup>3</sup> Both sexes on both diets were grouped to obtain these values.

<sup>4</sup> In the high fat and low fat diets there were isocaloric replacements of fat and carbohydrate. Although the term high fat diet has been used in the text, it should be noted that any differences observed between these two diets may equally well be due to the carbohydrate changes.



diets were employed in this investigation, differing in their fat content. In *immature female* birds receiving the two diets, statistically significant differences did not appear in any of the blood lipid constituents examined. A significant difference, however, did appear in *male birds* fed the two diets. This occurred in the cholesterol ester fraction, which was present to the extent of  $21.6 \pm 3.5$  mg. per cent in male birds fed a low fat diet and of  $42.1 \pm 6.1$  mg. per cent in those ingesting a high fat diet. Thus a difference of  $20.5 \pm 7.0$  mg. per cent exists between the concentration of cholesterol esters of the blood of male birds on low and high fat diets. This value is 2.9 times its standard error and hence statistically significant. The differences effected by these two diets upon all other blood lipid constituents were less than 1.8 times the standard error and hence not significant.

The effect of fat in the diet upon the blood lipids of the laying bird is more difficult to determine. Because of the extreme variability of the lipid levels, several samples were taken at intervals from a number of laying birds. Analysis of variance (10) showed that individual birds within each diet are significantly different with respect to their blood lipid levels and that therefore the preceding method of analysis would not be applicable to the data at hand. As may be noted from Tables IV and V the distributions of values of some individual birds are highly skewed, and consequently differences in means are of little significance.

In Tables IV and V it may be seen that some lipid constituents of laying birds fed the low fat diet are more variable than corresponding values for birds fed the high fat diet. To demonstrate this point coefficients of variability  $((\sigma \times 100)/M)$  were calculated for lipid values of birds that received four or more blood examinations.<sup>5</sup> When their variances were analyzed between and within diets, significant differences<sup>6</sup> between diets were found

<sup>5</sup> The first two values of Bird 78 (Table V) were not included in this or in subsequent calculations, since the bird was in pause at these times.

<sup>6</sup> The criterion used here is the  $F$  value of Snedecor (10), which is the ratio of the variances between and within classes. With the degrees of freedom available, an  $F$  value of over 5.59 would appear only once in twenty trials under conditions of random sampling. Higher  $F$  values are therefore probably not due to chance and may be termed significant. Thus the  $F$  values for free cholesterol (6.80), total fatty acids (8.54), neutral fat fatty acids (7.78), and for total lipids (9.16) indicate significant differences in

for free cholesterol and fatty acids. Similarly, analysis of variance of the extreme figures showed that the highest values of fatty acid fractions from birds fed the low fat diet were significantly higher than corresponding values obtained from birds fed a high fat diet.<sup>7</sup> These results suggest either that the abnormally small amount of fat in the low fat diet operated in some way to increase the variability of free cholesterol as well as the variability and maximum quantities of neutral fat in the blood, or that the abnormally large amounts of fat in the high fat diet operated to limit the action of whatever factor may normally be responsible for such fluctuations.

These effects of diet on the variability of blood lipid values were not found in males or immature females. This points to the existence of an interaction between ovarian activity and the diet such that the variability in the amounts of certain lipid fractions in the blood is reduced in the presence of a higher concentration of fat in the diet when the ovary is active. It may be postulated, therefore, that the substitution of fat for carbohydrate in the diets (high fat) modifies some part of the mechanism by which blood lipids are increased in response to ovarian activity.

*Relation of Ovarian Activity to Blood Lipid Levels*—Ovarian activity was considered from two points of view: (1) "production to date," which refers to the total number of eggs laid by the time of removal of the blood sample; (2) "intensity of production," which refers to the number of eggs laid per day during the period of active laying limited by 7 days preceding and 7 days after the blood was examined. Analysis of covariance (10) between these measurements of ovarian activity and the levels of any of the

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variability between the birds on the two diets. The  $F$  values for cholesterol ester and phospholipids, namely 3.47 and 4.20 respectively, are not significant and may be considered as having arisen by chance.

<sup>7</sup> Thus the  $F$  value for total fatty acids was 9.65, for neutral fat fatty acids 8.37, and for total lipids 9.22. These  $F$  values were calculated from the same degrees of freedom as above and so have the same standard of significance. The highest extremes for phospholipids had an  $F$  value of 4.75, while free and ester cholesterol had  $F$  values of less than 1.50. This indicates that the three latter fractions did not react to the dietary fat in the same way as the other constituents. Significant  $F$  values were not observed for any constituent when the lowest extremes were treated in the same manner.

blood lipid constituents failed to show any relationship so long as the bird was actively laying.

*Association of Blood Lipid Constituents of Laying Birds*—Coefficients of correlation between free and ester cholesterol, phospholipid, and neutral fat fatty acids were calculated by the method of covariance. Significant correlations were not obtained between ester and any other constituent, but free cholesterol was significantly correlated with phospholipid ( $r = 0.884$ ) and with neutral fat ( $r = 0.632$ ), and the two latter variables were significantly correlated ( $r = 0.671$ ). When birds on the two diets are considered separately, or when the correlation coefficients are calculated either between or within individual birds, these figures are not sufficiently affected to alter their meaning. Apparently the fluctuations in free cholesterol, phospholipids, and neutral fat are controlled in part by a single factor, whereas those in cholesterol ester are under the control of a different mechanism.

#### SUMMARY

1. The concentration of the various lipid constituents in the blood of the male and female bird was investigated in early age, puberty, and maturity.

2. No significant differences were found in the levels of cholesterol, phospholipids, or total fatty acids in the blood of *male* birds examined at various age periods from 71 to 276 days.

3. Lipid values comparable with those found in the male bird were observed in the *immature* female from 17 to 135 days of age.

4. A marked but variable increase in the lipid concentration of the blood occurs in birds actively engaged in *egg laying*. Values as high as 4719 mg. per cent for total lipids were found. The following constituents share in this increase: neutral fat, phospholipids, and free cholesterol. No significant changes were observed in esterified cholesterol.

5. Duration or intensity of egg production bore no relation to the increased level of the blood lipids provided the bird was in the laying state throughout the period of observation.

6. Oviposition is not the stimulus to the rise in the blood lipids, for a similar rise was found in birds examined during *puberty*.

7. The level of dietary fat had no effect on the concentration of the various blood lipid constituents of the immature female bird.

8. The level of dietary fat had no effect on the concentration of neutral fat, phospholipids, or free cholesterol in the male bird. Cholesterol esters, however, were somewhat increased when fat was substituted for carbohydrate in the diet.

9. Enormous concentrations of lipids may appear in the blood of the laying bird maintained on very low fat diets. Substitution of fat for carbohydrate in the diet of the actively laying bird had no effect on the cholesterol esters or phospholipids of the blood. The variability of neutral fat and free cholesterol is decreased in the presence of higher concentrations of ingested fat. This indicates an interaction between dietary fat and ovarian activity.

10. A high positive correlation exists among the phospholipid, free cholesterol, and neutral fat content of the blood of the laying bird. The trend of fluctuations of these lipid constituents in the laying state is in the same direction.

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## ACETALS IN THE SUGAR GROUP\*

### I. THE DIMETHYL ACETAL OF *d*-GALACTOSE

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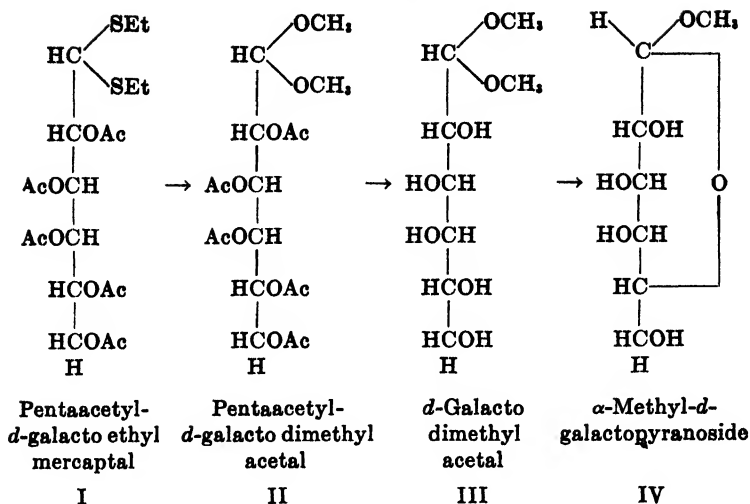
One of the objectives of our studies on the derivatives of *d*-galacturonic acid (1-3) has been to realize representative furanose derivatives. Certain control reactions with the parent sugar *d*-galactose have proved to be very helpful in conjunction with this work. This communication describes the synthesis of *d*-galacto dimethyl acetal (III) which was realized as follows:

Pentaacetyl-*d*-galacto ethyl mercaptal (I) was converted to the corresponding dimethyl acetal (II) by a procedure essentially identical to that employed by Pacsu and Green for the formation of glycofuranosides from the sugar mercaptals (4). Deacetylation of (II) produced the crystalline *d*-galacto dimethyl acetal (III) in good yields. Hydrolysis of the unsubstituted acetal (III) in a dry HCl-CH<sub>3</sub>OH mixture (after Fischer) led to the isolation of  $\alpha$ -methyl-*d*-galactopyranoside (IV).

The syntheses and transformations described in this paper are illustrated in the accompanying formulas.

The above control study on *d*-galactose was executed successfully in the autumn of 1936. In the interim Hudson and co-workers have reported on the synthesis of the crystalline *d*-arabo dimethyl acetal which they realized through an entirely different approach (5). The triacetyl  $\beta$ -methyl-*d*-arabinoside was treated with an acetylating mixture in the presence of dilute

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H<sub>2</sub>SO<sub>4</sub> or ZnCl<sub>2</sub> to produce the pentaacetyl-*d*-arabo methyl hemiacetal, which was converted to the corresponding chloro compound by the action of aluminum chloride. The action of silver oxide in methanol on the latter produced the tetraacetyl-*d*-arabo dimethyl acetal, which on deacetylation yielded the crystalline *d*-arabo dimethyl acetal. It appears from a search of the literature (6-8) that the *d*-arabo dimethyl acetal of Hudson and the corresponding *d*-galacto dimethyl acetal reported on herewith represent the first free sugar acetals. An extension of this study to *d*-xylose, *d*-glucuronic acid, and *d*-galacturonic acid, as well as biochemical studies on the acetals in the sugar group, will be reported on shortly.

#### EXPERIMENTAL

All analyses reported were made with the Pregl micromethods. The rotations were determined with a Franz Schmidt and Haensch polarimeter No. 52b with monochromatic light. The melting points were ascertained in a Thiele tube, the temperature being elevated at the rate of 2° per minute.

**Preparation of Pentaacetyl-*d*-Galacto Dimethyl Acetal**—4 gm. of pentaacetyl-*d*-galacto ethyl mercaptal prepared according to the method of Wolfrom and Morgan (9) were dissolved in 40 cc. of absolute methanol in a 3-necked round bottom flask equipped with

a condenser and stirrer. The solution was brought to the boiling point, whereupon 7.5 gm. of  $\text{HgCl}_2$  in 15 cc. of methanol and 2.0 gm. of  $\text{HgO}$  were added. After refluxing had proceeded for 1 hour, the white precipitate of  $\text{C}_2\text{H}_5\text{S}\cdot\text{HgCl}$  was removed and the boiling continued in the presence of  $\text{HgO}$  for an additional 20 minutes, during which time no more mercuric mercaptide separated. The  $\text{HgO}$  was removed and the filtrate concentrated to dryness under reduced pressure in the presence of cadmium carbonate. Extraction of the residue with warm chloroform removed the product. 20 cc. of methanol were then added to the chloroform solution, and  $\text{H}_2\text{S}$  gas was passed into the solution to remove the mercury salts. The hydrochloric acid produced from the  $\text{HgCl}_2$  was removed by shaking with  $\text{AgCO}_3$ . When this solution was concentrated, 2.5 gm. of the pentaacetyl-*d*-galacto dimethyl acetal separated in large diamond-shaped crystals. After being recrystallized from boiling water and dried over  $\text{P}_2\text{O}_5$  at  $25^\circ$  for 8 hours, the product showed the following analytical values.

$\text{C}_{18}\text{H}_{32}\text{O}_{12}$ . Calculated,  $\text{OCH}_2$  14.20; found,  $\text{OCH}_2$  14.10

*Melting Point*— $124.5\text{--}126^\circ$  (uncorrected)

*Rotation*— $[\alpha]_{589.3}^{25} = +20^\circ$  in methanol,  $c = 3.6\%$

*Preparation of d-Galacto Dimethyl Acetal*—The catalytic deacetylation of the pentaacetyl-*d*-galacto dimethyl acetal (II) was carried out with barium methylate, according to Isbell's procedure (10). 3 gm. of the acetylated acetal were suspended in 75 cc. of methanol and the mixture cooled to  $-4^\circ$ . To the mixture were added 2.5 cc. of 0.58 *N* barium methylate in methanol, and the mixture was maintained at  $0^\circ$  for 20 hours with occasional shaking. The barium methylate was decomposed by adding dilute sulfuric acid until the reaction mixture was neutral to methyl red, whereupon the solution was repeatedly concentrated under diminished pressure and taken up in methanol. When the methanol solution was cooled, the pure *d*-galacto dimethyl acetal crystallized in the form of needles, which after drying at  $78^\circ$  over  $\text{P}_2\text{O}_5$  (10 mm. pressure) in the Fischer pistol, showed the following analytical values.

$\text{C}_8\text{H}_{16}\text{O}_7$ . Calculated,  $\text{OCH}_2$  27.4; found,  $\text{OCH}_2$  27.1

*Fehling's Test*—Negative

*Melting Point*— $120\text{--}121^\circ$  (uncorrected)

*Rotation*— $[\alpha]_{589.3}^{25} = +16^\circ$  in water,  $c = 3\%$  (after 15 minutes). No change in rotation after 15 hours



**Hydrolysis of *d*-Galacto Dimethyl Acetal. Method I**—Fischer's conditions for glycoside formation (11) were observed for this hydrolysis. A 20 per cent solution of the *d*-galacto dimethyl acetal in 0.5 per cent dry HCl-CH<sub>3</sub>OH (in a sealed tube) was heated for 50 hours at 100°. The reaction product was handled in the usual manner and finally recrystallized from 95 per cent

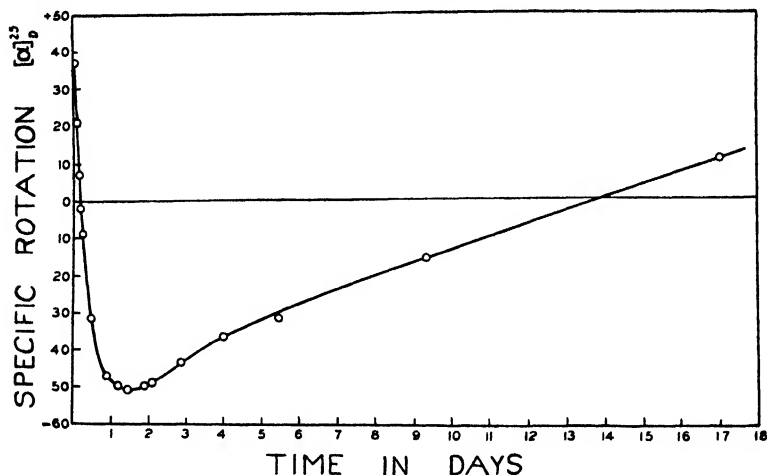


FIG. 1. Hydrolysis of *d*-galacto dimethyl acetal in dry HCl-CH<sub>3</sub>OH

ethanol. After the  $\alpha$ -methyl-*d*-galactopyranoside was dried under reduced pressure in the Fischer pistol, the following analytical values were realized.

C<sub>7</sub>H<sub>14</sub>O<sub>6</sub>. Calculated, OCH<sub>3</sub>, 14.7; found, OCH<sub>3</sub>, 14.7

*Fehling's Test*—Negative

*Melting Point*—105° (uncorrected)

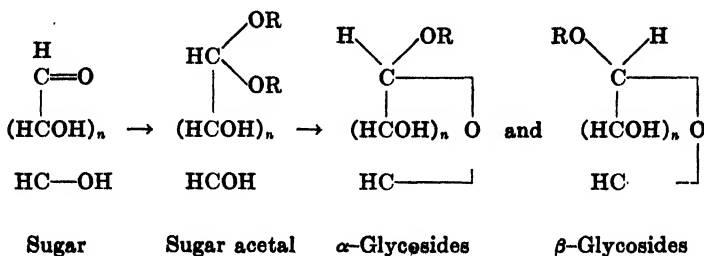
*Rotation*— $[\alpha]_{589.3}^{25} = +176^\circ$  in water,  $c = 3.0\%$ . (The accepted values are, m.p. 110–111° (corrected);  $[\alpha]_D = +178^\circ$  (6, 8))

**Method II**—This hydrolysis was carried out at room temperature (25°), as were those of Levene and coworkers (12). The hydrolysis of a 20 per cent solution of the *d*-galacto dimethyl acetal in a dry 0.5 per cent HCl-CH<sub>3</sub>OH mixture was followed polarimetrically. The values realized are presented in Fig. 1.

#### DISCUSSION

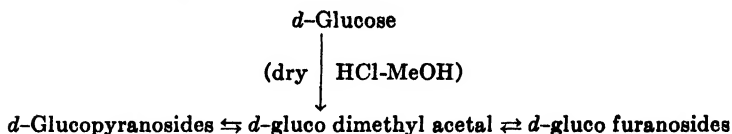
There has been considerable interest in the sugar acetals since Fischer (11) opened up this field in the course of his pioneer studies

on glycoside formation. Fischer originally postulated that the acetals should exist, as do their stable sulfur analogues, the thioacetals, and that they were intermediate substances produced in the course of glycoside formation as follows:



In 1914 Fischer pointed out that what he originally assumed to be the long sought dimethyl acetal was an isomeric mixture of glucosides (at the time termed  $\gamma$ -glucoside) which are now recognized to be the *d*-gluco methyl furanosides (13).

Fischer postulated essentially the following scheme, in which the equilibrium is shifted in the direction of pyranoside formation at elevated temperatures.<sup>1</sup>



The hydrolysis of the *d*-galacto dimethyl acetal in a dry HCl-CH<sub>2</sub>OH mixture (0.5 per cent HCl) conducted at 25° indicates, on the basis of the rotation values, that furanoside formation takes place rapidly and is followed by pyranoside production. The curve realized (Fig. 1) follows the general trend reported by Levene and coworkers (12) for glycoside formation in the sugar group.<sup>2</sup>

This is the first time that the unsubstituted dimethyl acetal of *d*-galactose has been obtained, although Levene and Meyer had reported some years previously (15) on the pentamethyl-*d*-galactose dimethyl acetal and the glucose and mannose ana-

<sup>1</sup> Green and Pacsu (4) have presented a more detailed scheme on glycoside equilibrium.

<sup>2</sup> The *d*-galacto methyl furanoside has, according to Haworth and coworkers (14), an  $[\alpha]_D$  of approximately  $-60^\circ$  in 1 per cent HCl-CH<sub>2</sub>OH.

logues. Certain biochemical studies on the sugar acetals are in progress and will be reported on shortly.

#### SUMMARY

1. The preparation of the pentaacetyl-*d*-galacto dimethyl acetal and of the *d*-galacto dimethyl acetal are described. This is the first time that these compounds have been obtained in a crystalline condition.

2. The *d*-galacto dimethyl acetal hydrolyzes to the  $\alpha$ -methyl-*d*-galactopyranoside under the conditions originally employed by Fischer for glycoside formation.

3. Polarimetric evidence is presented which indicates that when the *d*-galacto acetal is hydrolyzed (at 25° in dry HCl-MeOH mixture) furanoside formation takes place first and subsequently the more stable pyranosides are formed.

4. All compounds isolated were obtained in a crystalline condition and the physical constants and analyses are reported.

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# **ELECTROKINETIC ASPECTS OF SURFACE CHEMISTRY**

## **III. A COMPARISON OF THE MICROSCOPIC AND MOVING BOUNDARY METHODS OF ELECTROPHORESIS**

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(Received for publication, October 26, 1937)

It was shown by Loeb (1) that an approximate correspondence exists between the isoelectric points of gelatin or of egg albumin when in solution or adsorbed on the surfaces of microscopic particles. It remained for Abramson (2) to demonstrate a quantitative agreement between his own results for the electric mobility of serum albumin adsorbed on quartz surfaces and measured by the microscopic method of electrophoresis and the data of Tiselius (3) for solutions of freely dispersed serum albumin in U-tubes. Approximate agreement between the electric mobilities of adsorbed and dissolved egg albumin was also obtained but, in this case, it was noticed by Abramson that the isoelectric point of egg albumin is slightly shifted incidental to its adsorption. A recent reinvestigation of the electrokinetic properties of adsorbed egg albumin by Smith (4) and Moyer (5) has demonstrated that this divergence between the behavior of adsorbed and dissolved egg albumin is greater than had been originally indicated, the shift in isoelectric point being about 0.3 pH unit upward on the pH scale. Moyer also found that the shape of the electric mobility-pH curve of the adsorbed protein remains very nearly the same as in the dissolved state (5), except for a slight change in slope on the acid side. This was found true for egg albumin adsorbed on particles of quartz, glass, carbon, collodion, and mineral oil. Other, more polar surfaces, such as  $\text{SiO}_2$  and  $\text{Al}_2\text{O}_3$ , produced additional changes in slope from that obtained with the dissolved

\* John D. Jones Scholar, Biological Laboratory, Summer, 1937.

protein, although the isoelectric point underwent no further shift.

In addition to these comparisons of crystalline proteins, Howitt (6) has presented a series of measurements of the electric mobilities of various mammalian red blood cells, investigated by means of the moving boundary method. These agree very well with data of Abramson (7) on the red cells of the same species measured by the microscopic method. The value given by Abramson for the electric mobility of human erythrocytes has also been obtained by Moyer (5, 8), using horizontal microelectrophoresis cells, and likewise by Abramson, Moyer, and Voet (9), using the vertical microelectrophoresis instrument which they describe. Measurements of the electric mobilities of red cells are, at best, a difficult procedure and are limited to physiological conditions of pH and salt concentration. As shown by Kermack, M'Kendrick, and Ponder (10), the sedimentation rate and electric mobility of red cells or other large particles are diminished by the effects of crowding, so that experiments with suspensions concentrated enough to form definite boundaries may not yield correct values. In addition, corrections must be applied to the measurements in U-tubes for the force of gravity on the red cell (6), so that comparisons of this type are of less accuracy than comparisons based on the behavior of pure proteins.

Since the question of the absolute accuracy of the microscopic method is at stake, and in view of the divergence between the data for adsorbed and dissolved egg albumin, a careful reinvestigation of this problem seemed desirable. In this communication, data will be presented on the electric mobility of adsorbed serum proteins which are in complete agreement with the results of Tiselius for solutions of the same proteins measured by the moving boundary method under similar conditions.

### *Methods*

*Protein Preparations*—A slight modification of the method which Kekwick and Cannan (11) have used for egg albumin was employed. An equal volume of saturated sodium sulfate at 30° was added to normal horse serum (without preservative). After a day, the globulins were removed by centrifugation. 0.2 N sulfuric acid was then added cautiously to the albumin solution

with vigorous stirring until opalescence appeared. After several days the crystals were removed and redissolved in a volume of water equal to the original volume of serum. Anhydrous sodium sulfate was slowly added to this solution, with stirring, until recrystallization began. When complete, the crystals were separated and redissolved. The solution was dialyzed 2 days in running tap water and 3 days in distilled water until free from sulfate. The final solution was virtually colorless.

After the globulins were dissolved in water, the acidity was cautiously adjusted to pH 5.3. Anhydrous sodium sulfate was slowly added until precipitation occurred. This process was repeated twice again. The final solution was dialyzed as above until sulfate-free. After removal of the euglobulin by filtration, the pseudoglobulin was water-clear with a faint bluish opalescence. Both the albumin and pseudoglobulin were used a few days after their preparation and were stored in a refrigerator. Concentrations of these proteins were determined by drying samples at 110° and weighing.

*Electrophoresis Measurements*—Electric mobilities were determined with an Abramson horizontal microelectrophoresis instrument (a modification of a cell designed by Northrop and Kunitz (8, 12)). The techniques employed have been described elsewhere (5, 8). The instrument was the same as that used in experiments reported in previous papers of this series (5, 13). To correspond with the data of Tiselius, all measurements have been corrected to 20°.

*Preparation of Suspensions*—A standard amount of the same aqueous suspension of collodion as previously used (5) was placed in contact with a suitable quantity of the stock serum albumin solution. After a few minutes, the solution was diluted by addition of solutions of sodium acetate and acetic acid, so that the resultant buffer mixtures had an ionic strength of 0.02. Pseudoglobulin solutions were likewise placed in contact with collodion particles and, after a sufficient time had elapsed for coating, the calculated amounts of sodium acetate and acetic acid or  $\text{Na}_2\text{HPO}_4$  and  $\text{KH}_2\text{PO}_4$  were added, so that on dilution the resultant solution had an ionic strength of 0.1. The final protein concentrations varied between 0.1 and 0.2 per cent. Further addition of protein produced virtually no change in the results. pH measurements

were made with a quinhydrone electrode which was standardized against 0.1 N HCl, pH 1.08. The specific conductance of each solution was measured by a Wheatstone bridge.

#### EXPERIMENTAL

Fig. 1 presents our measurements of the electric mobilities of serum albumin adsorbed on collodion particles and also data of

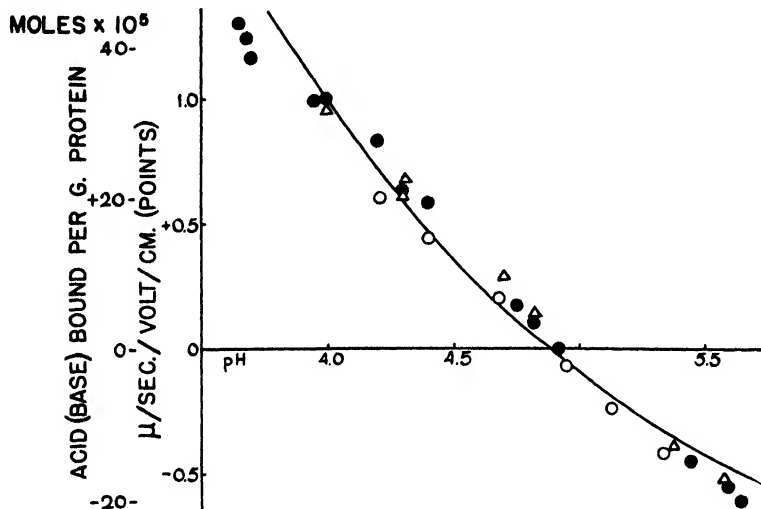


FIG. 1. The electric mobility of serum albumin as a function of pH, in 0.02 M acetate buffers at 20°. O data of Tiselius for the electric mobilities of dissolved horse serum albumin measured by the moving boundary method; ● data of Abramson for this protein adsorbed on microscopic quartz particles; Δ our own data for serum albumin adsorbed on collodion particles. The smooth curve is the titration curve drawn for comparison. There is no significant difference between the data for adsorbed and dissolved protein.

Abramson (2)<sup>1</sup> obtained with three preparations of serum albumin adsorbed on quartz particles. These microscopic measurements are compared with the results of Tiselius (3) for the electric mobility of dissolved molecules of serum albumin measured by the moving boundary technique. The smooth curve in Fig. 1 is not the "best" curve through the points but the titration curve for serum

<sup>1</sup> Personal communication from Professor Abramson.

albumin, in the absence of added salts, drawn for comparison. It will be noticed that our results fully confirm the conclusions of Abramson in regard to the identity of mobilities of adsorbed and dissolved serum albumin.

A recent paper by Tiselius (14) on the electrophoresis of serum globulins makes possible the comparison of adsorbed and dissolved pseudoglobulin. The results of such a comparison are shown in Fig. 2. Here too the agreement is highly satisfactory,

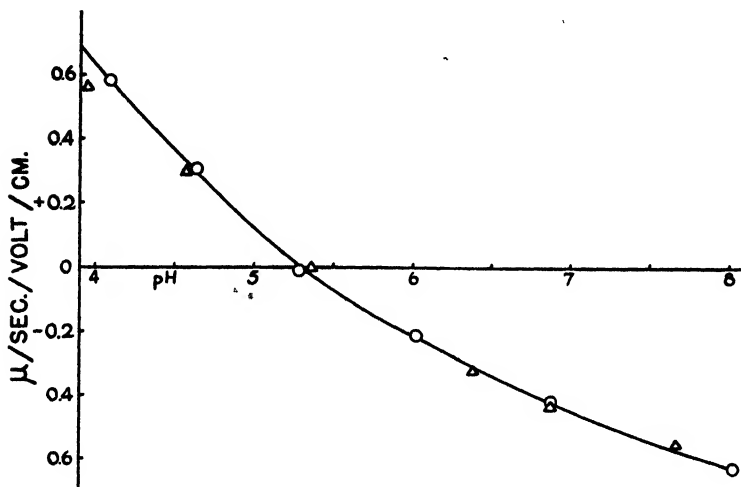


FIG. 2. The electric mobility-pH curve of pseudoglobulin at 20° in buffers of a constant ionic strength of 0.1. O data of Tiselius for the electric mobilities of dissolved horse serum pseudoglobulin measured in U-tubes; Δ mobilities of adsorbed pseudoglobulin measured by the microscopic method. The smooth curve has been drawn free-hand to fit Tiselius' data.

extending as it does from pH 4 to 8. It should be mentioned that the data below pH 5.5 were secured in acetate solutions and those above this point were measured in phosphate mixtures at the same ionic strength (0.1). Tiselius' data were also obtained under these conditions. These results substantiate the conclusions arrived at previously concerning the importance of ionic strength rather than total molarity in the determination of mobilities of protein and other surfaces (2, 5, 14, 15).



## DISCUSSION

The data presented in the experimental section give additional evidence that the investigation of adsorbed proteins reveals a true picture of the ionization of protein films. The microscopic method of electrophoresis, therefore, provides a simple and accurate method by means of which protein surfaces can be readily observed under various conditions. The demonstration that the ionization of adsorbed serum globulin is identical with that of dissolved molecules over the range of pH investigated emphasizes the value of this approach to the solution of the nature of the forces involved incidental to specific immunological reactions.

The data of Shibley (16) are of interest in this connection. Shibley has shown that denatured pseudoglobulin and pseudoglobulin adsorbed on collodion particles have the same electric mobility-pH curve within the limits of error. As his data for adsorbed pseudoglobulin were obtained at a different ionic strength (0.04 M glycine-phosphate-acetate buffers), his curve and ours cannot be compared quantitatively but their shapes and isoelectric points are nearly the same. The electrophoretic mobilities of sensitized bacteria were also identical with the data for pseudoglobulin (16). Later work of McCutcheon, Mudd, Strumia, and Lucké (17) indicates that in certain cases sensitized bacteria may be isoelectric at pH values different from the isoelectric point of any single constituent of normal serum.

## SUMMARY

Results obtained with the microscopic and moving boundary techniques of electrophoresis have been compared. It is shown that the electric mobility of horse serum pseudoglobulin adsorbed on microscopic collodion particles is the same, at any given pH value (in the region, pH 4 to 8), as the electric mobility of this protein in the dissolved state. These findings are discussed in connection with their immunological implications. Similar data of Abramson and Tiselius for horse serum albumin are confirmed. These results emphasize the accuracy and simplicity of the microscopic method.

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# DERIVATIVES OF GLUCURONIC ACID

## VIII. THE STRUCTURE OF BENZOYLGLUCURONIC ACID

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(Received for publication, July 28, 1937)

When benzoic acid is ingested by dogs, it is excreted in the urine partly in the form of hippuric acid and partly as a benzoyl ester of glucuronic acid. The detoxication of aromatic organic acids by conjugation with glucuronic acid is one of the important physiological mechanisms of man and certain animals. Benzoylglucuronic acid was first described by Magnus-Levy (1) who isolated it from the urine of sheep in the form of a dextrorotatory crystalline strychnine salt, from which he prepared an amorphous sodium salt. The structure of 1-benzoylglucuronic acid was assigned to this compound, though a critical survey of the original communication of Magnus-Levy reveals no direct chemical evidence in support of this hypothesis. This explanation of the constitution of benzoylglucuronic acid has been generally accepted until recent years.

Some time ago the question of the structure of benzoylglucuronic acid was reopened by Quick (2) who obtained the compound from the urine of dogs as a crystalline levorotatory substance. It was observed that solutions of benzoylglucuronic acid, when treated with traces of alkali, underwent a rapid change in rotation, a phenomenon which was attributed to mutarotation. On the basis of this evidence and because benzoylglucuronic acid reacted with dilute sodium cyanide solution, it was suggested that the aldehydic carbon atom of the conjugated derivative is free and that the compound is an ester substituted not on the 1st, but on one of the remaining carbon atoms of the hexoseuronic acid. This postulation has been questioned by Pryde and Williams (3) who favor the 1-benzoyl structure for benzoyl-

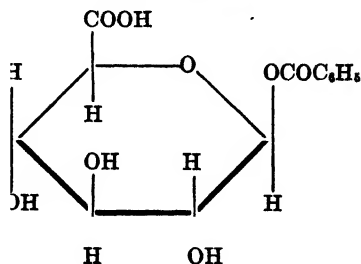
glucuronic acid. However, their interpretation of the experimental evidence in support of this contention has since been refuted by Quick (4). On the basis of the chemical evidence which is available at the present time it may be fairly stated that the exact constitution of benzoylglucuronic acid still remains obscure. It was thought possible, however, to establish the structure of this biologically important substance directly by synthesis.

The preparation of 1-bromo-2,3,4-triacetylglucuronic acid methyl ester has made possible the synthesis of derivatives of glucuronic acid substituted on the 1st or aldehydic carbon atom of the uronic acid (5). If benzoylglucuronic acid is an ester having the  $\beta$  configuration and substituted on carbon atom 1, as supposed by Magnus-Levy and by Pryde and Williams, then the triacetyl methyl ester of the naturally occurring derivative should be identical with the synthetic product obtained by condensing 1-bromo-2,3,4-triacetylglucuronic acid methyl ester with silver benzoate.

Natural benzoylglucuronic acid was isolated according to the method of Quick (2) and the methyl ester prepared by treating a cold alcoholic solution of the free acid with diazomethane. On acetylation of the latter derivative, triacetylmonobenzoylglucuronic acid was obtained in excellent yields. The substance crystallizes in the form of prismatic needles melting at  $145^{\circ}$  and having a specific rotation of  $-16.6^{\circ}$  in chloroform. Synthetic 1-benzoyl-2,3,4-triacetylglucuronic acid methyl ester was secured by condensing 1-bromo-2,3,4-triacetylglucuronic acid methyl ester with silver benzoate in boiling chloroform. When recrystallized from methyl alcohol, the synthetic derivative was found to be identical with triacetylmonobenzoylglucuronic acid methyl ester prepared from natural benzoylglucuronic acid. The crystalline structure, specific rotation, analysis, and melting point of both derivatives are identical and a mixed melting point of the two substances shows no depression.

Since 1-bromo-2,3,4-triacetylglucuronic acid methyl ester is a pyranose derivative having the  $\beta$  configuration (6), the substitution product, 1-benzoyl-2,3,4-triacetylglucuronic acid, may likewise be assigned the same ring structure and configuration. The latter derivative, which has been synthetically prepared, is identical with that derived from natural sources. The parent substance, levorotatory benzoylglucuronic acid, may therefore be

assigned the following structural formula in which the benzoyl group is attached to the 1st carbon atom.



In view of the evidence presented above pertaining to the structure of benzoylglucuronic acid, it is unlikely that the change in rotation which solutions of the sodium salt of benzoylglucuronic acid undergo in the presence of sodium hydroxide or the *initial* rapid change in rotation of such solutions in the presence of excess sodium cyanide can be attributed to mutarotation as postulated by Quick (2). An alternative explanation is therefore suggested; namely, that the phenomenon is the result of a complex series of reactions initiated by a migration of the benzoyl group, catalyzed by hydroxyl ions, from the aldehydic to one of the remaining carbon atoms of the hexose-uronic acid. The migration of benzoyl and acetyl groups of partially acetylated sugars in the presence of hydroxyl ions is of course a well known phenomenon and it is not unlikely that the benzoyl radical of monobenzoylglucuronic acid also migrates in the presence of hydroxyl ions.

In conclusion it is suggested that the amorphous dextrorotatory sodium salt of benzoylglucuronic acid of Magnus-Levy is in all probability the rearranged form of the levorotatory derivative described by Quick, for the former substance was isolated from an alkaline urine which had stood about for a considerable period of time (1). When certain precautions are taken, however, as Quick has shown, crystalline levorotatory benzoylglucuronic acid can be isolated in excellent yields.

#### EXPERIMENTAL

**Benzoylglucuronic Acid**—This substance was prepared from the urine of dogs which had been fed benzoic acid according to the directions of Quick (2). Contaminating hippuric acid was re-

moved from the crude benzoylglucuronic acid by extraction with ether in a Soxhlet extractor. Because of exceptional laboratory facilities for working in the cold, excellent yields of benzoylglucuronic acid were obtained. The urine of two dogs fed 35 gm. of benzoic acid yielded 17.5 gm. of crude benzoylglucuronic acid. After several recrystallizations from water the derivative showed a melting point of 184–185° (uncorrected).  $[\alpha]_D^{25} = -26.8^\circ$  in  $H_2O$  ( $c = 0.6$  per cent).

The specific rotation of an aqueous solution of the sodium salt of benzoylglucuronic acid prepared by adding 1 equivalent of 0.1 N NaOH to a weighed quantity of benzoylglucuronic acid at 0° was  $[\alpha]_D^{30} = -27.7^\circ$  ( $c = 1$  per cent). This value is in marked contrast to that of the amorphous sodium salt of benzoylglucuronic acid reported by Magnus-Levy,  $[\alpha]_D^{20} = +43.8^\circ$ .

*Benzoylglucuronic Acid Methyl Ester*—6.0 gm. of pure benzoylglucuronic acid were dissolved in 100 cc. of methyl alcohol and the solution cooled to  $-10^\circ$ . An ethereal solution of diazomethane, also at  $-10^\circ$ , was added in slight excess. The solvents were removed by distillation *in vacuo* and the residue dissolved in 300 cc. of boiling water. On cooling, crystals of benzoylglucuronic acid methyl ester separated. 4.5 gm. of substance were recovered. The compound melted at 190–191° (uncorrected).

$$[\alpha]_D^{25} = -16.3^\circ \text{ in } CH_3OH \text{ (} c = 1.5\% \text{)}$$

*Analysis*— $C_{12}H_{14}O_6COOCH_3$ . Calculated,  $OCH_3$  9.9; found, 10.1

Quick reported a melting point of 178–180° and  $[\alpha]_D^{20} = -25^\circ$  in  $H_2O$  for this derivative (2).

*Acetylation of Glucuronic Acid Methyl Ester*—3.0 gm. of benzoylglucuronic acid methyl ester were dissolved in a mixture of 12.5 cc. of freshly distilled anhydrous pyridine and 8.5 cc. of acetic anhydride at  $-5^\circ$ . After standing for  $1\frac{1}{2}$  hours at 0° the mixture was gradually warmed to room temperature. The solution remained clear and colorless. The solvents were removed by distillation *in vacuo* and the residue dissolved in 25 cc. of methyl alcohol. 3.7 gm. of prismatic needles melting at 143–145° were recovered.

After several recrystallizations from methyl alcohol the compound melted sharply at 145° (uncorrected).

$$[\alpha]_D^{25} = -16.6^\circ \text{ in } \text{CHCl}_3 \text{ (} c = 1.5\% \text{)}$$

*Analysis*— $\text{C}_{20}\text{H}_{22}\text{O}_{11}$ . Calculated. C 54.8, H 5.1,  $\text{OCH}_3$  7.1  
Found. " 55.1, " 5.3, " 7.1

*Synthesis of 1-Benzoyl-2,3,4-Triacetylglucuronic Acid Methyl Ester*—2.0 gm. of 1-bromo-2,3,4-triacetylglucuronic acid methyl ester were dissolved in 50 cc. of anhydrous alcohol-free chloroform and 3.5 gm. of anhydrous silver benzoate added. The mixture was refluxed for  $3\frac{1}{2}$  hours, filtered, and the chloroform evaporated from the filtrate *in vacuo*. The colorless syrupy residue was dissolved in 15 cc. of methyl alcohol. Crystals of monobenzoyl-triacetylglucuronic acid methyl ester separated immediately. After standing at  $0^\circ$  for 24 hours, 1.7 gm. of substance were filtered from the mother liquors. After several rapid recrystallizations from methyl alcohol, 1.4 gm. of substance melting at  $145^\circ$  were secured.  $[\alpha]_D^{25} = -16.9^\circ$  in  $\text{CHCl}_3$  ( $c = 1.5$  per cent).

When monobenzoyltriacylglucuronic acid methyl ester is crystallized slowly from methyl alcohol or from ether, the derivative is obtained in the form of large rhombs. The melting point of this crystalline form is apt not to be as sharp as that obtained when the derivative is crystallized rapidly from methyl alcohol.

Synthetic 1-benzoyl-2,3,4-triacetylglucuronic acid methyl ester is identical with the triacetyl ester derivative prepared from natural benzoylglucuronic acid as described above. A mixed melting point of the two substances showed no depression. The crystalline structure of the two preparations was identical and the specific rotations were likewise the same.

#### SUMMARY

1. The synthesis of 1-benzoyl-2,3,4-triacetylglucuronic acid methyl ester and its preparation from natural benzoylglucuronic acid are described.
2. The structure of benzoylglucuronic acid has been ascertained.

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## THE INFLUENCE OF PIMIENTO PIGMENTS ON THE COLOR OF THE EGG YOLK OF FOWLS\*

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(Received for publication, November 15, 1937)

The feeding of pimiento to fowls results in reddish orange pigmentation of the egg yolk, adipose tissue, skin, shanks, and beak (1, 2). The red pigment of the pimiento has been shown to be capsanthin (3), which is a dihydroxy monoketone. The pimiento pericarp has been found to contain zeaxanthin, cryptoxanthin (4), and  $\beta$ -carotene (5) in addition to capsanthin. Besides the four pigments mentioned, dried whole pimientos contain a little lutein (4) which is present in the large green stems.

It has been found (6) that ordinarily the xanthophylls of the egg yolk are lutein and zeaxanthin. The ratio present depends upon that present in the feed. When oil solutions of the single pigments, carotene, lycopene, and violaxanthin (7), were fed to white Leghorn hens, carotene and lycopene were found in traces at most, and violaxanthin not at all, in the egg yolk. Gillam and Heilbron (8) reported that the "carotene" fraction of the yolk pigments consisted largely of cryptoxanthin, with but a trace of carotene. Palmer and Kempster (9) found that annatto, which contains bixin, was without influence on the color of the adipose tissue of fowls.

The molecular structure of a carotenoid seems to exercise considerable influence on the deposition of pigments of this class. The work reported here was carried out in order to determine whether or not capsanthin itself is deposited in the egg yolk, and

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A preliminary report was presented before the Division of Biological Chemistry of the American Chemical Society at Chapel Hill, April, 1937.

if so, to what extent it is deposited compared with the ordinary xanthophylls.

#### EXPERIMENTAL

*Identification of Capsanthin in Egg Yolk*—Twenty-four single comb white Leghorn hens were placed in individual cages of a laying battery. Water and the following ration were kept before them until the yolks indicated that the hens had been depleted of carotenoid pigments.

	per cent
Ground white corn.....	43.5
"    wheat.....	18.0
Wheat bran.....	10.0
Flour wheat middlings.....	10.0
Meat and bone meal (50% protein).....	10.0
Dried skim milk.....	5.0
Limestone flour (marble).....	2.0
Salt flour.....	0.5
Cod liver oil.....	1.0

After depletion of carotenoids, ground dehydrated pimiento shells were added to the basal ration, a small amount at first, increased until 8 per cent of pimiento was reached. After the yolks had become uniform in color, the eggs were coated with paraffin oil and stored at 4°.

The yolks of twenty-two eggs were separated from the whites. The yolks, weighing 450 gm., were coagulated with ethyl alcohol, extracted with acetone, and purified according to Kuhn, Winterstein, and Lederer (6). The egg oil, although red, was centrifuged out and rejected. On standing in a separatory funnel over water, red crystals separated from the concentrated petroleum ether solution. A solution of a part of these crystals in carbon disulfide exhibited absorption maxima at 514 and 482 m $\mu$ . A solution of the crystals in 0.2 ml. of chloroform was placed in a 5 mm. absorption cell and 1 ml. of a 30 per cent solution of antimony trichloride in chloroform was added to it. The blue solution resulting exhibited an absorption band at 590 m $\mu$ , which soon disappeared.

On extraction of the petroleum ether solution of the pigment with 85 per cent methyl alcohol, most of the pigment was removed by the methyl alcohol. Therefore, it is evident that the greater part of the pigment was zeaxanthin, with but a small amount of cryptoxanthin or zeaxanthin ester.

Since the yolk color was dark reddish orange, zeaxanthin and cryptoxanthin could not be responsible for all of the color. From the color of the yolks one would expect capsanthin to be present, although, except for giving a blue coloration when concentrated HCl was added to an ether solution of the pigments, there were no positive data. In their examination of the carotenoids of human fat, Zechmeister and Tuzson (10) were unable to obtain crystalline capsanthin, even when it was added.

As capsanthin is extracted from its ether-petroleum ether (1:1) solution by 75 per cent methyl alcohol, while zeaxanthin and cryptoxanthin are not extracted to any extent at this concentration, this was expected to permit the concentration of any capsanthin present.

Yolks were extracted and purified as before. The pigment soluble in 90 per cent methyl alcohol was driven into ether-petroleum ether by dilution of the alcohol with much water and the alcohol washed out. The ether-petroleum ether solution was extracted with 75 per cent methyl alcohol, which extracted part of the pigment, but left the greater part in the upper layer. The pigment in the 75 per cent methyl alcohol was transferred to ether, the alcohol washed out, and the ether solution dried with anhydrous sodium sulfate. The ether solution was evaporated to dryness *in vacuo* on the water bath, leaving a salve-like residue.

A carbon disulfide solution of a part of the residue exhibited absorption maxima at 542 and 503  $m\mu$ . An ether solution of the residue underlain with concentrated HCl gave a greenish dark blue color at the junction of the two layers. A positive reaction was obtained with 25 per cent HCl also. Capsanthin is the only pigment present in the pimiento which gives this reaction.

*Feeding Mixtures of Capsanthin with Other Carotenoid Pigments*—In this experiment three lots of six hens each were used. One lot was fed a commercial all-mash ration which contained yellow corn and alfalfa. The remaining two lots were fed this ration plus different levels of pimiento. After the yolks had become uniform in color, the pigment content of the eggs was determined.

The amounts of pigment in the feed and egg yolks were determined by a colorimetric method (11), with alcoholic solutions of azobenzene as standards. The pigment content of several of the eggs is shown in Table I.

Based upon the feed consumption and the percentage egg production, at least 65 per cent of the ingested xanthophylls was deposited in the yolks of the first lot. In the lot with the smaller addition of pimiento, about 2 per cent of the total carotenoids was due to capsanthin. In the third lot, containing the larger addition of pimiento, up to 8.5 per cent of the total was due to capsanthin. Only about 1 per cent of the ingested capsanthin was deposited in the egg yolk.

TABLE I  
*Pigment Content of Egg Yolks*

Ration	Hen No.	Weight of yolk	Lutein + zeaxanthin per yolk	Capsanthin per yolk
		<i>gm.</i>	<i>mg.</i>	<i>mg.</i>
Commercial all-mash	1	20.0	0.600	
	5	21.5	0.572	
“ “ + 0.58%	13	20.5	0.800*	0.0125
dried pimiento	17	19.5	0.800*	0.0125
Commercial all-mash + 3.84%	7	20.5	1.200*	0.0359
dried pimiento	12	19.5	1.527*	0.0550

\* Calculated as lutein + zeaxanthin.

#### DISCUSSION

Since the dried pimiento was the only source of carotenoid pigments fed to the hens in the first experiment, and as it has been shown that the pimiento contains capsanthin, the behavior of the pigment in the phase test, its absorption spectrum, and positive HCl reaction show that the hen deposits capsanthin in the egg yolk. As the pigment extract was not saponified, it is also evident that this pigment is deposited in the yolk at least in part in the free state. The capsanthin esters would have remained in the ether-petroleum ether layer, and the ether solutions of its esters do not give a blue color with HCl.

Under the conditions of this experiment, from 2 to 8.5 per cent of the total carotenoids deposited in the yolk was capsanthin. Only about 1 per cent of the ingested capsanthin was deposited in the egg yolk. Gillam and Heilbron (8) found that from 3.3 to 9.5 per cent of the carotenoid content of the egg yolks which they examined was cryptoxanthin. Both pigments have one  $\beta$ -ionone ring with one hydroxyl group substituted in position

(5) in their molecules. The second ring of cryptoxanthin is  $\beta$ -ionone, while the open ring of capsanthin contains both one hydroxyl and one carbonyl group.

The two xanthophylls commonly found in egg yolks, lutein and zeaxanthin, are deposited to a much greater extent than capsanthin and cryptoxanthin. Both lutein and zeaxanthin have two hydroxyl groups, one in each ring. Since these two xanthophylls are deposited equally well, it does not seem to be of importance whether they contain substituted  $\alpha$ - or  $\beta$ -ionone rings.

While the exact structures of violaxanthin and taraxanthin are not known at present, both apparently contain two hydroxyl groups in one ring and either two in the other or else one hydroxyl and one carbonyl group. Two hydroxyl groups or one hydroxyl and one carbonyl group in both rings make a carotenoid as unavailable for deposition in the fowl as lycopene or the carotenes.

The annatto pigment, bixin, which also is not deposited by the fowl, contains a polyene chain, but no ring.

It would be of interest to know whether or not rhodoxanthin, with one carbonyl group in each ring, can be deposited by the fowl.

#### SUMMARY

1. When present in the ration of the hen, capsanthin is deposited in the egg yolk.

2. Capsanthin is deposited to about the same extent as cryptoxanthin.

3. In order for a fowl to deposit a carotenoid in the egg yolk or fat, it appears to be necessary that at least one ring of the molecule contain one, and only one, hydroxyl group.

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# CONVERSION OF URONIC ACIDS INTO CORRESPONDING HEXOSES

## IV. CATALYTIC REDUCTION OF THE METHYL ESTER OF DIACETONE *d*-GALACTURONIC ACID

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(Received for publication, November 20, 1937)

In previous communications<sup>1</sup> we have described the catalytic reduction of the methyl ester of 2,3,4-trimethyl  $\alpha$ -methyl-*d*-galacturonide and of the methyl ester of 2,3,4-triacetyl  $\alpha$ -methyl-*d*-galacturonide. In the case of simple uronic acids the conversion of the resulting glycosides into the free sugars is a simple procedure.

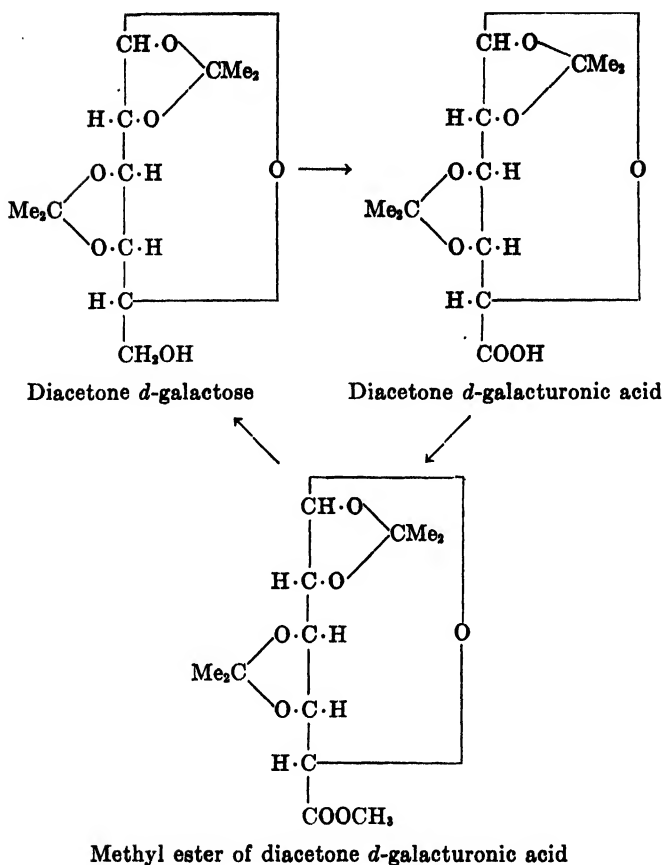
The situation is different in the case of aldobionic acids for the reason that the removal of the methoxyl group from carbon atom (1) of the resulting disaccharide glycoside requires treatment often sufficiently drastic to cleave the disaccharide linkage. It was thought that the acetone derivatives of uronic acids might retain the acetone residue during the process of reduction and thus might lead to acetone hexoses, which then could be converted readily into the corresponding free sugars. This expectation was actually realized.

The methyl ester of diacetone *d*-galacturonic acid was reduced to diacetone *d*-galactose under the same conditions as previously described. The stages leading from diacetone *d*-galactose back to the same substance are given in the accompanying formulæ.

This method now is being applied to the conversion of certain free aldobionic acids into the corresponding disaccharides.

<sup>1</sup> Levene, P. A., Tipson, R. S., and Kreider, L. C., *J. Biol. Chem.*, **122**, 199 (1937-38). Levene, P. A., and Christman, C. C., *J. Biol. Chem.*, **122**, 203 (1937-38).





## EXPERIMENTAL

*Attempted Catalytic Reduction of Diacetone d-Galactose*—5 gm. of copper chromite catalyst were added to a solution of 5 gm. of diacetone *d*-galactose ( $n_D^{25} = 1.4653$ ) in 100 cc. of absolute methanol. This mixture was placed in a high pressure reduction apparatus and a hydrogen pressure of 3000 pounds per sq. inch was applied. The temperature was now slowly increased to  $175^\circ$ . At this temperature the pressure rose to 4300 pounds per sq. inch. After the reaction had proceeded during 5 hours at this temperature, the whole apparatus was allowed to cool for 18 hours.

The reaction mixture was removed, some charcoal added, and

the catalyst separated by filtration. The filtrate was concentrated to dryness and the resulting sirup distilled under a high vacuum, giving three fractions all having a refractive index of  $n_D^{25} = 1.4653$ . Yield 4.8 gm. The material had the following specific rotation

$$[\alpha]_D^{25} = \frac{-6.67^\circ \times 100}{2 \times 5.800} = -57.5^\circ \text{ (in chloroform)}$$

and was therefore unchanged diacetone *d*-galactose.

*Preparation of Methyl Ester of Diacetone d-Galacturonic Acid*—10 gm. of dry diacetone galacturonic acid<sup>2</sup> (m.p. 156°) were slowly added to a cold solution of 2 gm. of diazomethane in 100 cc. of dry ether. The reaction was instantaneous and was accompanied by the rapid evolution of nitrogen. The mixture, after standing overnight at room temperature, still contained diazomethane, as indicated by the faint yellow color.

The solution was filtered and the filtrate evaporated under diminished pressure to a thick sirup which could not be induced to crystallize. It was therefore distilled under diminished pressure. The whole of the substance boiled at 133° (bath temperature) and 0.17 mm. pressure. Yield 9.7 gm. Four fractions were collected, each of which had a refractive index of  $n_D^{25} = 1.4622$ . The substance had the following specific rotation.

$$[\alpha]_D^{25} = \frac{-9.72^\circ \times 100}{2 \times 5.200} = -93.4^\circ \text{ (in chloroform)}$$

It is soluble in acetone, chloroform, benzene, heptane, ethyl and methyl alcohols, and ether but is practically insoluble in pentane or water.

The composition of the substance agreed with that for the methyl ester of a diacetone hexuronic acid.

$C_{11}H_{20}O_7$ .	Calculated.	C 54.13,	H 7.0,	OCH <sub>3</sub> 10.76
	Found.	" 54.29,	" 7.11,	" 10.79

*Catalytic Reduction of Methyl Ester of Diacetone d-Galacturonic Acid*—5 gm. of copper chromite catalyst were added to a solution of 5 gm. of the methyl ester of diacetone *d*-galacturonic acid in 100 cc. of absolute methanol. This mixture was placed in a high

<sup>2</sup> Niemann, C., and Link, K. P., *J. Biol. Chem.*, **104**, 195 (1934).

pressure reduction apparatus and a hydrogen pressure of 3000 pounds per sq. inch was applied. The temperature was now slowly increased to 175°. At this temperature the pressure rose to 4300 pounds per sq. inch. After the reaction had proceeded during 5 hours at this temperature, the whole apparatus was allowed to cool for 18 hours.

The reaction mixture was removed and the catalyst separated by filtration. The filtrate was treated with charcoal and again filtered in order to remove the last traces of catalyst. A colorless sirup was obtained upon concentration of this filtrate. Yield 4.5 gm.

The sirup was now distilled at a bath temperature of 120° and a pressure of 0.17 mm. Only one fraction was collected. Yield 4.3 gm. This material was then redistilled and four fractions were collected at a bath temperature of 115° and a pressure of 0.13 mm. The first fraction was taken for analytical data. It had a refractive index of  $n_D^{25} = 1.4655$  and a specific rotation of

$$[\alpha]_D^{25} = \frac{-5.04^\circ \times 100}{2 \times 4.240} = -59.4^\circ \text{ (in chloroform)}$$

The substance had a composition agreeing approximately with that calculated for a diacetone hexose.

4.227 mg. substance: 8.595 mg. CO<sub>2</sub> and 2.975 mg. H<sub>2</sub>O

C<sub>12</sub>H<sub>20</sub>O<sub>6</sub>. Calculated, C 55.35, H 7.7; found, C 54.44, H 7.9

## A DIRECT ACIDIMETRIC MICROTITRATION METHOD FOR CALCIUM

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(Received for publication, October 1, 1937)

The theoretical advantages of the acidimetric<sup>1</sup> methods for the microtitration of calcium have been pointed out by several authors (1-5). In actual practise, however, oxidimetric methods have gained more favor owing to their relative simplicity. Whereas in the acidimetric methods the titrimetric equivalent is obtained indirectly, in the oxidimetric methods this equivalent is obtained directly. Difficulties are encountered that are common to both the acidimetric and oxidimetric methods, owing to the instability of the standard titrating solutions. This is caused by the changing titer of the standard alkali<sup>2</sup> as well as the standard oxidimetric solutions at 0.01 N strength. In contrast, the titer of standard acids of this strength is extremely stable. An acidimetric method which would eliminate the use of standard alkali by employing a direct titration would therefore eliminate the undesirable features of the present methods and yet retain

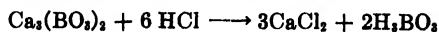
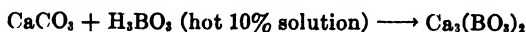
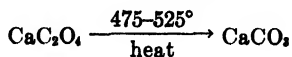
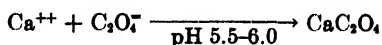
<sup>1</sup> The term *acidimetric* is employed in this paper as a convention, when a base is titrated with a standard acid. The term *oxidimetric* is used when a reductant is titrated with a standard oxidizing agent. In both cases the titration may be direct or indirect, depending upon the particular method. In the *direct titration* an equivalent amount of standard solution is used. In the *indirect titration* an excess of standard solution is employed and this excess is titrated back with a standard alkali in the acid-base systems and with a standard reductant in the oxidation-reduction systems.

<sup>2</sup> With special precautions against absorption of carbon dioxide from the air and by carefully lining the container with pure paraffin 0.01 N standard alkali is fairly stable. In actual practise, however, it is not always possible to observe these precautions. Thus, in the every-day experience of many microanalysts, who are often pressed for time, a change in the titer of 0.01 N alkali is often encountered.

the theoretical advantages of the acidimetric and the simplicity of the oxidimetric procedures.

In order to obtain the desirable features of the present titrimetric methods, and eliminate the use of unstable standard solutions, the possibility of employing boric acid for the microestimation of calcium was investigated. Boric acid is used as the acid to trap ammonia in the microestimation of ammonia (6, 7). It is also used to trap the bases in the microestimation of total base (8). The trapped ammonia or base is titrated to the pH of a pure boric acid solution with standard acid. This titration represents the titrimetric equivalent of the ammonia or base. Thus, a direct titration is used instead of the customary indirect titration and the use of standard alkali is completely eliminated. After examination of various experimental conditions it was established that a hot solution of 10 per cent boric acid will dissolve calcium carbonate in a few seconds and that the titrimetric equivalent of the dissolved calcium may be obtained by adding a standard acid until the pH of the boric acid solution is reached. The quantitative precipitation of calcium as the oxalate and its conversion to the carbonate were carried out essentially the same way as previously described by one of the authors and his coworkers (9).

The principle of the method as finally developed is presented below.



Calcium ion is precipitated as the oxalate, which is then converted to the carbonate at a temperature of 475–525°. The carbonate is dissolved in a hot solution of 10 per cent boric acid. The solution is diluted and titrated directly with a 0.01 N standard acid to the pH of a pure boric acid solution of similar strength. This titration represents the amount of calcium in equivalents.

This method has a combination of desirable features, all of which are not present in any of the hitherto available micro-methods for calcium. The procedure is simple and accurate. A

direct titration is employed with a sharp end-point. Oxalate ion is employed in the washing fluid and thus loss of the calcium oxalate precipitate through solution is minimized. Only one washing is necessary, as adsorbed oxalate ions are volatilized during the conversion of the oxalate to the carbonate.

### *Method*

#### *Reagents—*

0.01 N acid. 100 cc. of a carefully prepared solution of 0.1 N hydrochloric or sulfuric acid are diluted with double distilled water to 1000 cc. This solution is kept in an automatic burette calibrated to 0.01 cc. The 0.01 N acid is stable for years if carefully handled.

Indicators. 0.04 per cent bromocresol purple prepared according to Clark (10) and a mixture of methyl red and methylene blue prepared according to Patterson (11) as follows: 100 cc. of 0.02 per cent methyl red and 30 cc. of 0.1 per cent methylene blue. This mixture is diluted to 500 cc. The proportion of methyl red and methylene blue may be changed to suit the operator.

Ammonium oxalate reagent. A saturated solution of c.p. ammonium oxalate is prepared at 40–50° and allowed to cool to room temperature. The clear supernatant solution is used.

Boric acid solution. 10 gm. of boric acid are dissolved in 100 cc. of distilled water, by heating. This solution is supersaturated at room temperature and consequently it is necessary to dissolve any boric acid that precipitates on standing by heating just before use. This solution is used while hot.

0.5 per cent ammonium oxalate. 0.5 gm. of c.p. ammonium oxalate is made up to 100 cc. with distilled water.

### *Procedure*

To 2.0 cc. of solution (containing about 0.1 to 0.4 mg. of calcium ions) in a 10 or 15 cc. Pyrex tube, 1 cc. of ammonium oxalate and 1 drop of bromocresol purple are added. This is washed down by 2 cc. of water and mixed. The pH is adjusted to the gray color which is between the yellow and purple of the indicator. The contents of the tube are allowed to stand for 1 hour or longer, after which it is centrifuged at about 2000 revolutions per minute for 10 minutes. The supernatant liquid is decanted or carefully

aspirated with a drawn out tube and rubber bulb without disturbing the precipitate. The precipitate is suspended in 3 cc. of 0.5 per cent ammonium oxalate and centrifuged for 5 to 10 minutes. The supernatant liquid is then carefully aspirated or decanted. The washed precipitate is dried at 100–110° and heated in a muffle furnace or sand bath at 475–525° for 20 to 30 minutes. After this the tube is placed in a boiling water bath and 0.5 cc. of the hot boric acid solution is added. The precipitate dissolves in about 1 to 2 minutes completely. The solution is diluted to 3 cc. with distilled water and 1 to 2 drops of Patterson's indicator added. This solution is then titrated with 0.01 N acid to the pH of a pure solution of boric acid of such concentration. (The titration back to the original pH of boric acid is most easily carried out by adding indicator to a control tube in which 0.5 cc. of boric acid is diluted to approximately 4.0 cc.) This represents the titrimetric equivalent of calcium.

*Calculation—*

1.0 cc. 0.01 N acid used = 0.2 mg. Ca

$$\text{Mg. per 100 cc.} = \frac{\text{cc. acid used} \times \text{equivalent weight} \times 100}{\text{cc. sample}}$$

*Procedure for Blood Serum—*The method is directly applicable to fresh serum except that at least 4 hours should be allowed for precipitation of the calcium oxalate.

#### DISCUSSION

The optimum temperature for the conversion of calcium oxalate to the carbonate is between 475–525° (12). In order to heat the calcium oxalate to this temperature range, in a short time, it is necessary to keep the temperature of the muffle furnace<sup>3</sup> higher. The actual temperature of the muffle furnace in most of these experiments was between 560–600°. With such a temperature the conversion of the oxalate to the carbonate is completed in 10 to

<sup>3</sup> The muffle furnace is essentially an air bath where temperature exchange is relatively slow. This takes place in part by radiation from the sides of the furnace and partially by direct heat transfer from the heated air. Both of these processes are much slower than direct contact with a liquid or finely powdered solid.

15 minutes. With a muffle furnace temperature of 490–530° this conversion takes at least 3 hours. One may substitute ordinary glass tube for Pyrex with careful temperature control provided the softening temperature of the glass is not reached. Aside from the undesirable effect of softened glass the calcium oxalate precipitate may be heated to above 525° and thus form some calcium oxide. This, however, does not influence the results, as the acidimetric equivalent of calcium oxide is identical with calcium carbonate. An insufficient heating period or a temperature much below 475° will prevent complete conversion of the calcium oxalate to the carbonate and thus give rise to results that are too low. In cases of incomplete conversion the precipitate will not dissolve completely in the hot boric acid.

The temperature as well as the concentration of boric acid influences its degree of dissociation. It is advisable, therefore, when a precision of a high order is required to standardize both of these factors carefully. In the present study, however, no special effort was made to standardize these conditions, as the precision of the method is satisfactory for our present purposes under the conditions described. As a rule 5 to 10 minutes elapsed between the dilution of the dissolved calcium carbonate and its titration. Thus, at the end of the titration the titrated solution is practically at room temperature. The difference between the volume of the pure boric acid solution (used for color matching) and the titrated solution was usually between  $\pm 0.5$  to  $\pm 1.0$  cc.

It is important to use an approximately 10 per cent solution of boric acid, as in more dilute solutions difficulties will be encountered in dissolving the calcium carbonate. Once the carbonate has dissolved, however, the solution may be diluted and better conditions for the titration thus obtained. In measuring out the boric acid it is advisable to preheat the pipette which is used for measuring and thus prevent precipitation of boric acid in the pipette.

### *Results*

Representative results of known solutions are presented in Table I. It may be observed that the maximum error of estimation is within 1 drop (0.04 cc.  $\simeq$  0.008 mg. of Ca) of the theoretically expected titration. The average error of 71 determinations



was  $-0.0006$  mg. The average deviation of 71 determinations is  $\pm 0.0030$  mg. Comparative results on blood serum by the Kramer-Tisdall (13) oxidimetric and the boric acid procedure are given in Table II. It is seen that excellent agreement is obtained by the two methods. The average difference between nineteen comparative estimations was  $+0.25$  per cent of the Kramer-

TABLE I  
*Determination of Calcium in Inorganic Solutions*

Ca present	Ca found	Ca present	Ca found
mg.	mg.	mg.	mg.
0.100	0.098	0.300	0.301
0.100	0.100	0.300	0.306
0.100	0.104	0.300	0.300
0.200	0.202	0.400	0.396
0.200	0.200	0.400	0.402
0.200	0.204	0.400	0.408
0.200	0.196		

TABLE II  
*Determination of Calcium in Serum*

The values are expressed in mg. per 100 cc.

Permanganate method	Boric acid method
9.3	9.3
9.3	9.4
9.5	9.5
9.2	9.1
9.3	8.9
9.4	9.6
10.1	10.2
9.4	9.3
10.2	10.3
10.3	10.4

Tisdall values, while the average agreement between nineteen pairs (one titrated oxidimetrically, the other acidimetrically) was  $\pm 1.57$  per cent of the Kramer-Tisdall values.

#### SUMMARY

1. A method is described for the acidimetric microestimation of calcium by a direct titration.

2. This is accomplished by precipitating calcium as the oxalate, which is then converted to the carbonate at 475–525°. The carbonate is dissolved in a hot solution of 10 per cent boric acid. The solution is diluted and titrated directly with a 0.01 N standard acid to the pH of a pure boric acid solution of similar strength. The titration represents the amount of calcium in equivalents.

3. This procedure retains the theoretical advantages of the acidimetric methods, employs only standard acid (which is stable) in a direct titration, and eliminates the use of standard alkali whose titer is difficult to keep at 0.01 N strength.

4. This method is directly applicable to blood serum.

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## THE DETERMINATION OF ASCORBIC ACID IN PLASMA; A MACROMETHOD AND MICROMETHOD

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Since the work of Tillmanns, Hirsch, and Hirsch (1), Harris and Ray (2), and Bessey and King (3), 2,6-dichlorophenol indophenol has been widely used for the determination of ascorbic acid in biological materials. Usually the oxidized dye has been titrated into a definite volume of strongly acid solution containing the unknown amount of ascorbic acid, and the persistence of the faintest pink for 30 seconds has been taken as the end-point. Because the titration in this procedure is carried to the point where no further reduction of the dye occurs in 30 seconds, it does not quantitatively differentiate between the rapid reduction of the dye by ascorbic acid and the slower reduction by other reducing substances. In whole blood filtrates, tissue extracts, and urine, reducing substances with slower action may be present in such amount as to cause inconstant end-points or false high values. Titration of the unknown solution into a fixed volume of the dye is no more satisfactory, as the end-point of complete disappearance of the dye is not sharp and the longer time occupied by the titration permits reduction of the dye by other reducing substances. To determine the reduction of 2,6-dichlorophenol indophenol by ascorbic acid alone, Emmerie and van Eekelen (4) have removed other reducing substances by precipitation with mercuric acetate. This procedure may permit the accurate determination of ascorbic acid but is time-consuming and requires large samples. Finally, any titration method requires the use of an accurately standardized dye solution.

When the titration procedure is applied to plasma, as described by Pijoan and Klemperer (5), the relatively small volume of dye

solution used in the titration and the faint color of the end-point introduce considerable error unless 1 to 2 cc. of plasma are used. Even with 2 cc. samples of plasma the error of the method is great for plasmas of low ascorbic acid content.

(The purpose of this paper is to describe a procedure for the determination of the ascorbic acid concentration in blood plasma which permits the detection of reduction of 2,6-dichlorophenol indophenol by substances that under the conditions reduce the dye more slowly than does ascorbic acid and which at the same time permits an accurate analysis from as little as 0.1 cc. of plasma.) A subsequent paper will report the application of the method to the determination of ascorbic acid concentration in whole blood and other tissues.

(The principle underlying the procedure has been discussed by Rosen and Evelyn (6). The decrease in the concentration of oxidized dye produced by the addition of an amount of plasma that is insufficient to cause complete reduction of the dye is measured by means of a photoelectric colorimeter. Observation of the rate of fading of the color with time permits study of the reaction velocities and thus the detection of reduction due to substances acting at a slower rate than ascorbic acid.) The method also eliminates the subjective reading of an end-point and the need of an accurately standardized dye solution. (Visual colorimetry for the procedure is unsatisfactory because of the difficulty of maintaining a stable series of standards and the longer time necessary for matching colors.)

In such strongly acid media as are used in the usual titration procedure (5, 7), the rate of fading of the dye is enough to cause an appreciable error at the concentrations of oxidized dye prescribed by our method. In the titration procedure, in which complete reduction of the dye is almost instantaneous until the end-point is reached, this error of fading is minimized. (In the procedure reported here, error from fading of the dye is prevented by adding such an amount of sodium acetate to the dye solution that the pH of the final dye-metaphosphoric acid filtrate of plasma is 4.1. This pH is not low enough to cause significant fading of the dye, and yet is not so high as to result in the immediate reduction of the dye by the reducing substances other than ascorbic acid that are present in the plasma. The oxidation of reduced

ascorbic acid other than by 2,6-dichlorophenol indophenol is prevented by the addition of cyanide to the blood immediately upon collection (5), by the acidity of the metaphosphoric acid plasma filtrate before the addition of the acetate-dye solution (7), and by the short time required for the procedure.)

### *(Macrodetermination)*

*(Apparatus)*—The photoelectric colorimeter described by Evelyn (8) has been used.<sup>1</sup> The Evelyn light filter No. 520, which transmits from 494 to 562  $m\mu$  with maximum at 514  $m\mu$  and mean at 520  $m\mu$ , gives a satisfactory change in galvanometer reading with change in concentration of the oxidized dye which in acid solution has a maximum absorption at 518  $m\mu$ .

#### *Reagents—*

Potassium oxalate solution, 20 per cent.

Potassium cyanide solution, 5 per cent.

Metaphosphoric acid solution, 6 per cent. This should be made up fresh at least every 2 weeks and stored in the ice box. The progressive change of meta- to orthophosphoric acid is accompanied by a decrease in pH which may cause rapid fading of the dye.

2,6-Dichlorophenol indophenol solution. Approximately 2.5 mg. per 100 cc. A few crystals may be dissolved without weighing in a small amount of distilled water at 85–95°, filtered, cooled, and diluted so that the blank analysis described under "Procedure" gives a galvanometer reading of 50 to 55 when the reading for distilled water is set at 100.<sup>2</sup> This solution keeps at least 3 weeks if stored in a dark bottle in the ice box.

Sodium acetate solution. 4.53 gm. of  $\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$  made up to 100 cc. with distilled water and brought to pH 7.0 with 0.26 cc. of 0.5 M acetic acid (9). If standardized acetic acid is not available, the pH of this solution may be adjusted by adding dilute acetic acid a few drops at a time, testing small aliquots with brom-thymol blue. To this solution should be added a few drops of toluene to insure its clarity on standing.

<sup>1</sup> The method is applicable for use with any equally accurate photoelectric colorimeter.

<sup>2</sup> This procedure for making a 2.5 mg. per cent dye solution may be used with any photoelectric colorimeter when the galvanometer reading for a blank analysis of such a solution is known.

Indophenol-acetate solution. Equal volumes of sodium acetate and 2,6-dichlorophenol indophenol solutions. Enough for several days may be made up at a time and kept in the ice box.

*Procedure*—Collect 4 to 5 cc. of venous blood in a test-tube containing 1 drop each of 5 per cent potassium cyanide and 20 per cent potassium oxalate. Shake immediately. Centrifuge and measure 2 cc. of plasma into a test-tube. Add 2 cc. of distilled water and 4 cc. of 5 per cent metaphosphoric acid. Shake gently and filter. The filtrate must be crystal-clear.

Set the galvanometer at the center setting<sup>3</sup> that gives a reading of 100 with a blank dye solution as described below but which has been completely reduced by an excess of ascorbic acid. Once this center setting has been found, it need not be determined again so long as the physical properties of the colorimeter remain constant. The constancy of the colorimeter may and must be established at each analysis by checking the center setting for distilled water; *i.e.*, the setting that gives a galvanometer reading of 100 when the proper filter and a colorimeter tube containing distilled water are in place.

Measure 4 cc. of the indophenol-acetate solution into one of the colorimeter tubes. Add 4 cc. of filtrate, stir, and immediately insert the tube into the colorimeter and note the galvanometer reading 30 seconds after the addition. Then observe the galvanometer readings 1, 2, and 3 minutes after the addition to be sure that no significant reduction of the dye due to a more slowly acting reducing substance is occurring<sup>4</sup> (see Curve 2, Fig. 1).

Run a blank as follows: Measure 4 cc. of the indophenol-acetate solution into another colorimeter tube, add 4 cc. of 2.5 per cent metaphosphoric acid, stir, and read immediately.

*Calculation*—Because 2,6-dichlorophenol indophenol solutions have an ascorbic acid equivalent and because the volumes of the sample solutions in the method described are constant, the con-

<sup>3</sup> The center setting is the galvanometer reading when there is no tube in the colorimeter (8).

<sup>4</sup> No significant further reduction has been noted in the many plasmas analyzed in this laboratory. If such reduction should occur, extrapolation of the curve after 30 seconds to zero time should give a more accurate ascorbic acid value.

centration of ascorbic acid in a sample solution is given by the equation

$$C = K (\log G_s - \log G_b)$$

where  $G_s$  is the galvanometer reading for the sample solution,  $G_b$  is the galvanometer reading of a similar dye solution containing no ascorbic acid, and  $K$  is a constant depending upon, first, the proportionality between the galvanometer reading and transmission of light through the colorimeter (8); second, the applicability of the laws of Lambert and Beer; third, the nature of the dye solution and filter used; and fourth, the volume of the sample solutions. The validity of the constant  $K$  and its numerical value for each sample of dye and each photoelectric colorimeter used are conveniently determined by a few determinations on carefully measured samples of varying concentrations of crystalline ascorbic acid dissolved in 2.5 per cent metaphosphoric acid. Using the photoelectric colorimeter in this laboratory, we have found the value of  $K$  on seven lots of dye from two manufacturers to be  $0.086 \pm 0.003$ . This variation in  $K$  does not result in a significant error in plasma determinations.

When the initial volume and the subsequent dilution of the plasma sample in the procedure described are taken into consideration, the mg. of ascorbic acid per 100 cc. of plasma are obtained by multiplying  $K$  by 100. We have found it convenient to construct an alignment chart for determining the ascorbic acid concentration in mg. per cent from the galvanometer readings of the blank and of the sample.

The dependence of the ascorbic acid concentration on the difference between the logarithms of the two galvanometer readings eliminates the need for an accurately standardized dye solution and makes the location of the center setting of the colorimeter unimportant, so long as it is the same for both the blank and the plasma filtrate tubes. However, the reading for complete reduction of the dye must be known, and  $G_s$  must never reach it, if the total reduced ascorbic acid present is to be determined. For this reason, it is convenient so to adjust the galvanometer that the reading of a completely reduced dye sample solution is 100. With the galvanometer so set, an excess of dye in the dye-plasma fil-



trate solution is indicated as long as the galvanometer reading is less than 100.

In the procedure described a dye solution that gives a blank reading of 50 provides the necessary excess of dye with plasma ascorbic acid concentrations up to 2.5 mg. per cent and therefore provides a range satisfactory for determinations on normal and scorbutic subjects. If ascorbic acid concentrations greater than 2.5 mg. per cent are anticipated, the concentration of the dye solution should be increased. Such higher concentrations of dye are not desirable routinely, as they diminish the sensitivity of the determination for low ascorbic acid concentrations owing to the logarithmic relation between the concentration of the dye and the galvanometer reading.

#### *Microdetermination*

*Apparatus*—Photoelectric microcolorimeter (10). If Filter 520 used in the macrodetermination is too opaque, Filter 520-M, which transmits from 456 to 610  $m\mu$  with maximum at 525  $m\mu$ , may be used.

Microcolorimeter cell equipped with glass plunger (11) whose height was adjusted so that it would be just below the surface when the cell contained 1.1 cc.

Micropipette with a modified Shohl tip (12), equipped with a No. 20 needle and calibrated to contain 0.1 cc. and to deliver 0.15 cc.<sup>5</sup>

Davies tubes (13). 8 cm. of glass tubing whose internal diameter is 2.5 mm., with a tightly fitting rubber cap at each end.

*Reagents*—The reagents and precautions are the same as for the macroprocedure except:

Cyanide-oxalate solution. 3 per cent potassium cyanide; 20 per cent potassium oxalate.

2,6-Dichlorophenol indophenol solution. The blank analysis

<sup>5</sup> Made by the Macalaster-Bicknell Company, Cambridge, Massachusetts. It is convenient to have additional calibrations to contain 0.08 and 0.06 cc., so that occasional small samples of blood need not be discarded. However, determinations on less than 0.1 cc. of plasma are not recommended as a routine because of the small difference between galvanometer readings of the blank and sample.

should give a galvanometer reading of 80 to 85 when distilled water reads 100. The concentration is about 1.2 mg. per 100 cc.

**Sodium acetate solution.** 1.51 gm. of  $\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$  made up to 100 cc. and adjusted to pH 7.0 with 0.11 cc. of 0.5 M acetic acid (9).

**Indophenol-acetate solution.** Equal volumes of these solutions.

**Procedure**—Prepare Davies tubes by filling a number of them with cyanide-oxalate solution, draining quickly and drying in the oven for 15 minutes at  $105^\circ$  before use. Collect 0.2 to 0.3 cc. of capillary blood in a prepared Davies tube. Close both ends of the tube with rubber caps and centrifuge. Remove the cap above the plasma and carefully transfer 0.1 cc. of plasma to a 15 cc. conical centrifuge tube, using the special pipette to facilitate removal of the plasma. With the same pipette add 0.15 cc. of distilled water, thus washing out the pipette. Add 0.2 cc. of 5 per cent metaphosphoric acid. Stir and centrifuge for at least 5 minutes or until the supernatant fluid is clear.

Set the galvanometer at the center setting obtained when the completely reduced blank dye solution reads 100 (see the macro-procedure).

Measure 0.8 cc. of indophenol-acetate solution into a microcolorimeter cell. With a fine tipped pipette that is accurately calibrated to deliver 0.3 cc., remove the supernatant fluid without contamination by the precipitate and add exactly 0.3 cc. to the indophenol-acetate solution in the microcell. Immediately stir, place the plunger in position, insert the cell into microcolorimeter, note the galvanometer reading 30 seconds after the addition, and then observe the readings at 1 and 2 minutes as described under the macroprocedure.

Run a blank as follows: Measure 0.8 cc. of indophenol-acetate solution into the microcell, add exactly 0.3 cc. of 2.5 per cent metaphosphoric acid, stir, and note the galvanometer reading immediately.

**Calculation**—The principles are identical with the macrodetermination. The value of  $K$  for the dye samples and photoelectric colorimeter used was found to be 0.028 with Filter 520-M and 0.023 with Filter 520. Conversion of the concentration of ascorbic acid in the sample to mg. per cent in plasma requires multiplication of  $K$  by 1500.

*Results of Determination of Ascorbic Acid in Plasma by  
Methods Presented*

Table I gives comparisons of the ascorbic acid concentration of plasmas determined by the titration method described by Pijoan and Klemperer (5) and the macro- and microprocedures described here. All the determinations on each patient were carried out on the same sample of plasma. The agreement between the values determined by the titration and the macro-photocolorimeter methods is good. A large series of determina-

TABLE I

*Determinations of Reduced Ascorbic Acid of Plasma with Photoelectric  
Colorimeter Compared with Titration*

Patient	Titration	Photocolorimeter	
		Macro	Micro
	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>
C. C.	1.3	1.2	1.0
M. M.	1.5	1.5	1.5
G. C.	1.0	0.9	1.1
H.	0.3	0.3	
R. B.	1.1	1.0	1.1
E. F.	0.1		0.2
M. S.	1.4		1.4
H. D.	1.1		1.0
M. S.	0.5		0.4
M. G. H.	0.4		0.2
A. C.	0.4		0.2
C. M.	0.2		0.1

tions by these two methods shows that the slightly lower values given by the photoelectric colorimeter are consistently found. We attribute this difference to the errors inherent in the titration procedure. The agreement between the macro- and microdeterminations (Tables I and II) is satisfactory. An error of 0.2 mg. per cent in plasma concentration is not as yet of clinical significance, even when applied to low plasma values. The data in Table II indicate that capillary blood does not vary markedly from venous blood in plasma ascorbic acid concentration and also that the collection of microsamples is not accompanied by oxida-

tion of the ascorbic acid. Table III shows the recoveries from addition experiments by both methods.

## DISCUSSION

*Fading of 2,6-Dichlorophenol Indophenol*—Variations in the rate of disappearance of the color of 2,6-dichlorophenol indophenol in

TABLE II  
*Ascorbic Acid Concentration of Simultaneous Venous (Macro) and Capillary (Micro) Samples*

Patient	Macro	Micro
	<i>mg. per cent</i>	<i>mg. per cent</i>
J. Q.	1.3	1.1
M. C.	0.9	0.9
P. H.	0.9	0.7
J. F.	1.1	0.9
C. F.	1.3	1.1

TABLE III  
*Recoveries of Ascorbic Acid Added to Plasma*

Ascorbic acid originally present	Ascorbic acid added	Ascorbic acid found (a)	Ascorbic acid calculated (b)	Error $\frac{100(a-b)}{b}$
<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	
Macro				
1.08	0.82	1.92	1.91	+0.5
1.54	0.61	2.09	2.15	-2.9
1.54	0.41	1.92	1.95	-1.6
Micro				
0.76	1.02	1.95	1.78	+9.6
0.76	0.82	1.49	1.58	-5.7
0.63	0.60	1.27	1.23	+4.5
0.63	0.40	0.96	1.03	-6.8

a metaphosphoric acid solution and also in buffered solutions of increasing pH are shown in the lower curves of Fig. 1. The higher the pH, the slower is the fading, so that at pH 4.1 the dye is stable for at least 5 minutes. That the fading is not due solely to reduction of the dye is shown by the failure of various oxidizing agents to restore the color, whereas dye at pH 5.0 which has been reduced by shaking with zinc dust when al-

lowed to stand in air spontaneously reoxidizes with reappearance of color.) Curves 1 and 2 show the velocity of reduction of the dye in dye-plasma filtrate solutions. The determination represented in Curve 2 was made as described in the macromethod, the pH being 4.1. The determination represented in Curve 1 was made with the addition of water, instead of the sodium acetate

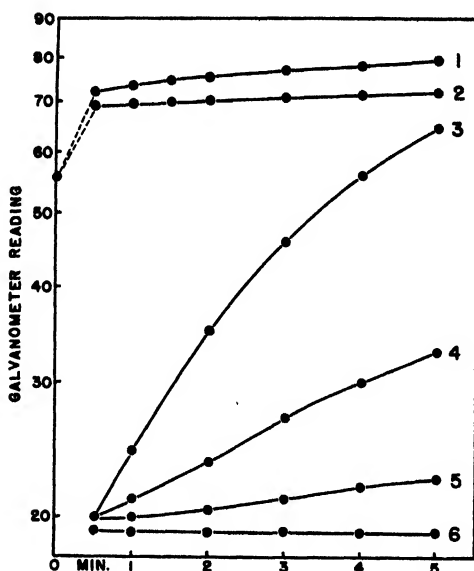


FIG. 1. Fading of 2,6-dichlorophenol indophenol. (a) 0.02 mm dye in metaphosphoric acid filtrates of plasma; Curve 1 pH 2; Curve 2 pH 4.1. (b) 0.064 mm dye in (Curve 3) 2.2 per cent  $\text{HPO}_4$ , pH 1.5; (Curve 4) phosphate buffer, pH 1.9; (Curve 5) phosphate buffer, pH 2.6; (Curve 6) acetate-metaphosphoric acid mixture used in macrodetermination, pH 4.1. In this and in Fig. 2 the galvanometer reading is plotted on a logarithmic scale, since the concentration is proportional to the negative log of the galvanometer reading.

solution, to the dye solution and, therefore, at a pH of 2. It is noted that the rate of fading in Curve 1 after 30 seconds is more rapid than in Curve 2. This may account for the slightly higher ascorbic acid value in Curve 1 as compared to Curve 2.

The rate of fading of 2,6-dichlorophenol indophenol in acid solution is apparently determined not only by the pH of the

medium but also by the concentration of the reduced form. For example, during 5 minutes 74 per cent of the color disappeared from a 0.04 mm solution of oxidized dye in 1.8 per cent metaphosphoric acid, whereas during the same time only 19 per cent of the color disappeared from a solution containing the same concentrations of oxidized dye and metaphosphoric acid but containing also 0.06 mm per liter of the reduced dye as the result of the reduction of the oxidized form by ascorbic acid. This stabilization is not due to the oxidized ascorbic acid, for reversibly oxidized ascorbic acid obtained by shaking reduced ascorbic acid with norit (14) has no stabilizing effect. Moreover, the presence of reduced dye prepared by shaking a faintly acid solution of the oxidized dye with zinc dust results in similar stabilization of the oxidized dye in acid solution.

*(Reaction of Ascorbic Acid with 2,6-Dichlorophenol Indophenol—*Even in the presence of relatively large amounts (0.1 mg.) of ascorbic acid, the reduction of the dye by the ascorbic acid is complete within the 20 to 25 seconds required for drainage of the pipette and stirring, as is indicated by constant galvanometer readings when the system is buffered at pH 4.1 or higher so that no destruction of the dye occurs. Neither the increase in pH to 4.8 nor the magnitude of excess 2,6-dichlorophenol indophenol affects appreciably the point of equilibrium. To study the influence of the presence of oxygen on the reaction, Thunberg type tubes were blown to fit the colorimeter and the dye added from the bulb after triple evacuation and washing with nitrogen. Results under these conditions were similar to experiments run in air.)

*Interfering Substances—*The determination of ascorbic acid by the quantitative reduction of 2,6-dichlorophenol indophenol depends on the extreme rapidity of the reaction in acid solution. Many other substances will reduce the dye but at a considerably slower rate, notably those of the general type  $R-SH$ , such as cysteine and reduced glutathione. These substances reduce the dye more rapidly with increasing pH and it was thought that with the system buffered at pH 4.1 a correction would have to be made for these more slowly acting compounds by noting the rate of disappearance of the red color after the first half minute, when all the ascorbic acid has been oxidized, and by extrapolating to zero time. However, reaction velocity curves on the plasma filtrates

under the conditions prescribed in the methods have given no evidence of reduction by such substances (see Curve 2, Fig. 1). Moreover, it has been found that over a wide range of concentrations of both reduced ascorbic acid and either cysteine or reduced glutathione (0.01 to 0.20 mg. in 10 cc.) the percentage error is insignificant, except at very small concentrations of ascorbic acid, if the half minute readings are taken to represent only as-

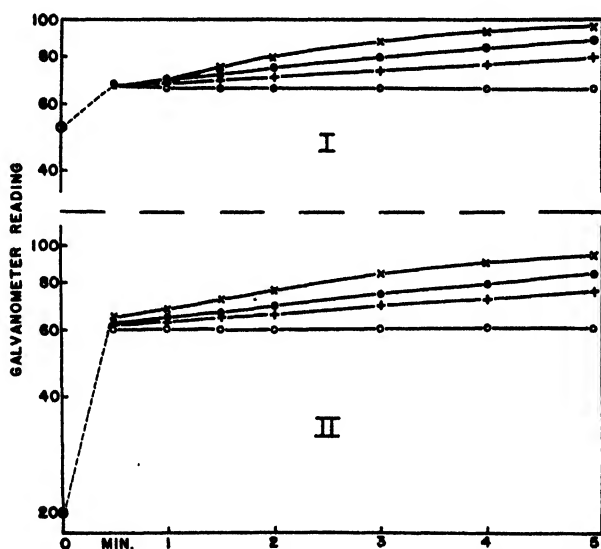


FIG. 2. Effect of cysteine on the determination of ascorbic acid at pH 4.1. Experiment I, 0.5 cc. of 1.0 mM dye added to 9 cc. containing 0.05 mg. of ascorbic acid and varying amounts of cysteine; Experiment II, 0.2 cc. of 1.0 mM dye added to 9 cc. containing 0.01 mg. of ascorbic acid and varying amounts of cysteine. X denotes 0.20 mg. of cysteine hydrochloride; ● 0.10 mg. of cysteine hydrochloride; + 0.05 mg. of cysteine hydrochloride; O 0.00 mg. of cysteine hydrochloride.

corbic acid. Fig. 2 gives curves for ascorbic acid-cysteine solutions reacting with 2,6-dichlorophenol indophenol. The gentle slope after the first half minute is the reduction due to cysteine. The rate depends on the concentrations of both the cysteine and the oxidized dye, but the latter has been varied with the ascorbic acid concentration to leave approximately the same excess of oxidized dye in all systems. Extrapolation of the cysteine part

of the curve to zero time gives more accurate recoveries of ascorbic acid but over the range of concentrations in these experiments results in a correction of less than 5 per cent.

While no special precautions were taken to eliminate traces of iron from the cysteine-ascorbic acid-indophenol systems, the addition of iron (0.01 mm per liter) had no effect on the rate of reduction of the dye.

#### SUMMARY

A study of the velocity of reduction over 30 second intervals of 2,6-dichlorophenol indophenol by metaphosphoric acid filtrates of plasmas has been made. A procedure which accurately measures this reduction over the first 30 seconds and permits detection of the presence of more slowly acting reducing substances has been described for the determination of the ascorbic acid concentration of plasma. The procedure reduces the errors due to fading of the dye and reading of the end-point that are inherent in the ordinary titration of ascorbic acid with 2,6-dichlorophenol indophenol. As written, it is directly applicable to the Evelyn photoelectric colorimeter. With the microapparatus satisfactory results can be obtained from as little as 0.1 cc. of plasma.

We wish to thank Dr. A. B. Hastings and Dr. A. T. Shohl for their helpful suggestions and discussion of this work with us and Dr. F. Klemperer for the determination of the pH of various solutions.

*Addendum*—Since this paper was submitted, we have seen the work of Meunier (15) who has made observations which are in agreement with those reported here.

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# REDUCTION OF CERTAIN SULFUR COMPOUNDS TO HYDROGEN SULFIDE BY THE INTESTINAL MICROORGANISMS OF THE DOG

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A number of papers have dealt with hydrogen sulfide formation from various sulfur compounds by the agency of specific cultures. Among the more recent ones may be cited those of Muesow and Paine (1), Zorkendorfer (2), Tarr (3), and Starkey (4). Most investigators of the effect of specific strains of bacteria on sulfur compounds are in agreement that whereas  $\text{H}_2\text{S}$  is easily produced by many microorganisms from compounds representing an intermediate state of oxidation of the sulfur, completely oxidized forms (sulfates and sulfonates) are, as a rule, not reduced to  $\text{H}_2\text{S}$ . Kochman (5), however, reports formation of  $\text{H}_2\text{S}$  from  $\text{Na}_2\text{SO}_4$  by the microorganisms of both cat and rabbit feces; Muesow and Paine (1) found  $\text{H}_2\text{S}$  production in certain cases from  $\text{K}_2\text{SO}_4$  (but not from  $\text{Na}_2\text{SO}_4$ ), while Zorkendorfer (2) reports some production of  $\text{H}_2\text{S}$  from sulfates in the intestine. Other investigators have reported negative results with sulfates.

In the present investigation, no attempt has been made to work with pure cultures, since our interest lay mainly in determining the practical results of bringing various sulfur compounds into contact with the mixed bacteria of the intestinal mucosa. In all cases the fresh mucosa of the small intestine of dogs was removed and hashed very shortly after death. A weighed amount of the mucosa (50 gm.) was placed in a  $29 \times 300$  mm. test-tube. To this was added an amount of the sulfur compound to be tested corresponding in sulfur content to 0.002 mole (0.480 gm.) of cystine.

When necessary to produce an approximately neutral solution, the substance was added as the sodium salt. This tube, provided with inlet and outlet tubes for aeration, was connected to a second tube containing 50 cc. of an ammoniacal solution of cadmium nitrate ( $3.5 \text{ gm. of } Cd(NO_3)_2 \cdot 4H_2O + 25 \text{ cc. of concentrated } NH_3 + 25 \text{ cc. of } H_2O$ ) for absorption of the hydrogen sulfide. A third tube, containing 5 N  $H_2SO_4$ , served to remove ammonia from the stream of air. This was necessary, since from two to four such groups were always run in series at one time in order to produce as uniform results as possible. It was usually found practicable to run a series of three or four determinations at one time with different portions of the same sample of intestinal mucosa. In each series, a charge of *l*-cystine was used to provide a reference substance, while other sulfur compounds were used in the other groups. Thus variations in the content of microorganisms in different lots of intestinal mucosa were always standardized against one reference substance (*l*-cystine) and the data obtained were calculated as ratios of the degree of decomposition of *l*-cystine, the latter being taken as unity (see Table I).

The apparatus was placed in a constant temperature room regulated at  $38^\circ$  and air was slowly bubbled through the entire train for 48 hours. The cadmium sulfide produced from each substance was then filtered, washed, covered with excess standard 0.100 N iodine solution, and acidified. The excess iodine remaining after the reaction with the hydrogen sulfide was titrated with 0.100 N sodium thiosulfate. The sulfur compounds used were of a satisfactory degree of purity, as evidenced by sulfur and nitrogen determinations. *l*-Cystine was prepared from human hair; *i*-cystine, by boiling *l*-cystine in hydrochloric acid until racemization was complete; dibenzoylcystine, by the method of Morrow (6); cystine hydantoin and cysteic acid hydantoin, as described by Andrews and Andrews (7); cystine phenylhydantoin and cysteic acid phenylhydantoin, as described by Andrews and Andrews (8); and cysteic acid, by oxidation of cystine with bromine (9). The taurine and methionine were commercial samples of practically theoretical purity. The inorganic salts and the free sulfur were of ordinary c.p. grade. Dibenzoylcystine and all compounds containing a sulfonic acid group were used in the form of their sodium salts.

In Table I are summarized the data obtained. Column 1 represents the actual percentage decomposition of each individual substance as compared with the percentage decomposition of cystine shown in Column 2. Decomposition is expressed in terms

TABLE I  
*Effect of Intestinal Bacteria on H<sub>2</sub>S Production from Various Sulfur Compounds*

Compound	Percentage decomposition		Average corrected ratio (l-cystine = 1.00) (3)
	Compound (1)	l-Cystine control (2)	
dl-Cystine	46.4	39.8	
	44.2	48.0	1.03
Dibenzoylcystine	4.3	38.0	
	4.9	49.1	0.00
Cystine hydantoin	12.0	25.4	
	30.6	49.1	0.47
“ phenylhydantoin	0	25.4	
	0	69.8	0.00
Cysteic acid hydantoin	0	19.9	
	0	55.8	0.00
“ “ phenylhydantoin	0	19.9	
	0	48.0	0.00
“ “	2.2	25.4	
	2.0	58.0	0.00
Taurine	8.2	58.0	
	7.7	39.8	0.08
Methionine	11.1	38.0	
	16.5	55.8	0.21
Sodium sulfate	6.0	68.9	
	6.9	60.9	0.03
“ sulfite	15.3	66.6	
	11.9	41.5	0.18
“ thiosulfate	18.6	66.6	
	15.4	41.5	0.26
Free sulfur	43.9	55.8	
	35.3	60.9	0.65

of that percentage of the charge represented by the hydrogen sulfide produced. Since each separate train of experiments was conducted with a different sample of fresh mucosa, the variations in percentage decomposition of cystine represent variations in the activity of microorganisms in different lots of mucosa. It will

be noted that in every instance the same *l*-cystine control was used for two different experimental substances; the same percentage decomposition for cystine therefore always appears twice in Table I.

Table I also contains (in Column 3) the results of recalculating the data in terms of the ratios of the percentage decomposition of *l*-cystine, the latter being taken as unity. In this calculation a blank correction for the hydrogen sulfide produced from the mucosa itself has been applied. Three such blank determinations, expressed in cc. of 0.1 N iodine solution, amounted to 2.5, 6.0, and 3.0 cc. The average (3.8 cc.) has therefore been used as a blank correction on all determinations. All figures in Column 3 of Table I have been so corrected. In spite of the fact that a number of different dogs were used for these experiments, these ratios are remarkably constant.

With certain compounds (cystine phenylhydantoin, cysteic acid hydantoin, and cysteic acid phenylhydantoin) even less hydrogen sulfide was obtained than in the blank determination. This inhibiting action on the part of the two phenyl derivatives is sufficiently plausible but to observe such an effect on the part of cysteic acid hydantoin is somewhat surprising. Other experiments with these substances, not recorded in Table I, confirmed this result. A degree of decomposition corresponding roughly to the blank was obtained with dibenzoylcystine, while, as would be expected, neither the sulfonic acid group (cysteic acid and taurine) nor the hydantoin ring (cystine hydantoin) showed any inhibiting properties.

Although no significant difference was observed between the degree of decomposition of *l*- and of *i*-cystine, we have further tested the possibility of preferential bacterial action on the individual isomers by treating a 2 gm. sample of *i*-cystine with a correspondingly larger amount of mucosa as described above for several days. After decomposition had reached nearly 50 per cent, as indicated by titration of the cadmium sulfide produced, the mixture of mucosa was acidified and filtered. The cystine was then purified by three recrystallizations and dried. It was found to be optically inactive. It must be concluded that the mixed microorganisms of the intestine of the dog show no special preference for the natural isomer of cystine. Assuming that similar results would apply to the intestine of the rabbit *in vivo*,

it would seem that the differences in metabolic oxidation reported by du Vigneaud, Craft, and Loring (10) for  $\alpha$ - and *l*-cystine definitely represent a real difference in intracellular oxidation rather than the result of a greater degree of production and subsequent oxidation of hydrogen sulfide from the natural isomer. This suggests the possibility that the differences in oxidation reported by du Vigneaud, Craft, and Loring may have been considerably dulled by production and metabolic oxidation of hydrogen sulfide from these compounds, and that if their results could have been corrected for this factor, still more striking differences between the rate of metabolic oxidation of the isomers of cystine might have been apparent.

Similar possibilities arise in comparing hydrogen sulfide production from *l*-cystine with that from *dl*-methionine. Du Vigneaud, Loring, and Craft (11), as well as others, have reported that oral administration of *dl*-methionine and of *l*-cystine produces similar degrees of oxidation of the sulfur administered as measured by the production of urinary sulfate. The data in Table I show a definitely lower degree of decomposition of methionine and thus imply a correspondingly more rapid metabolic oxidation for this amino acid. The ability of the normal organism to oxidize rapidly the sulfur of methionine is further emphasized by the greater speed of its absorption from the small intestine (12).

The effect of intestinal microorganisms on cysteic acid and taurine is of interest in connection with the studies of White, Lewis, and White (13). These authors report production of a limited amount of urinary sulfate after oral administration of cysteic acid and of considerably more sulfate after oral administration of taurine. They suggest that intestinal production of hydrogen sulfide may be responsible for a portion of this sulfate. The data in Table I indicate some decomposition of taurine but not of cysteic acid and, if we may assume that the effect of intestinal microorganisms in rabbits is similar to that in the dog, lend support to the conclusion of White, Lewis, and White that there is some evidence of sulfate production from cysteic acid in the metabolism of the rabbit.

The results obtained with inorganic sulfur compounds as well as with free sulfur correspond to those previously reported by most investigators (*cf.* (14)), particularly as regards the very slight evidence of sulfide production from sodium sulfate.

## SUMMARY

The ability of a mixture of microorganisms from the mucosa of the small intestine of the dog to produce hydrogen sulfide from the following compounds has been quantitatively compared: *l*-cystine, *i*-cystine, dibenzoylcystine, cystine hydantoin, cystine phenylhydantoin, cysteic acid hydantoin, cysteic acid phenylhydantoin, cysteic acid, taurine, methionine, sodium sulfate, sodium sulfite, sodium thiosulfate, and free sulfur.

Of the above, the following gave no evidence of producing hydrogen sulfide: cystine phenylhydantoin, cysteic acid, cysteic acid hydantoin, and cysteic acid phenylhydantoin. The apparent production of hydrogen sulfide from sodium sulfate was negligibly small.

A definitely inhibiting action was exhibited by cystine phenylhydantoin, cysteic acid hydantoin, and cysteic acid phenylhydantoin.

No appreciable difference in the rate of decomposition of *l*- and *i*-cystine was observed. Furthermore, no evidence was obtained that these microorganisms decompose the *l* isomer in preference to the *d* form.

The possible bearing of these and other findings on the interpretation of metabolic studies of sulfur compounds is discussed.

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# THE BIOCHEMISTRY OF MAGNESIUM DEFICIENCY\*

## I. CHEMICAL CHANGES RESULTING FROM MAGNESIUM DEPRIVATION

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The study of the importance of magnesium for animal nutrition has lagged far behind that of other inorganic elements present to any considerable extent in the body. Not until 1932 was it conclusively demonstrated that it is a nutritional essential, when Kruse, Orent, and McCollum (2) were able to prepare a diet analyzed as containing 0.18 mg. of Mg per 100 gm. of food. They fed this diet to rats newly weaned weighing 40 to 50 gm. and demonstrated for the first time the spectacular series of symptoms which are characteristic of a severe magnesium deficiency. The syndrome as described by these authors is as follows: (1) A vasodilatation and hyperemia develop in the peripheral vascular bed, during which reddening of all exposed skin appears in 3 to 5 days and lasts 10 to 12 days. (2) In about 18 days the animals become extremely hyperexcitable and any startling noise throws them into a tonicoclonic convulsion. (3) Most of the animals die in the first convulsion. Those that survive develop trophic changes in the epidermal structures and an edema of the extremities.

The conclusion was reached that the hyperexcitability caused by magnesium deficiency is a new form of tetany distinguished by a

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† This communication is constructed from a thesis submitted to the Graduate Division of the University of California by Elma V. Tufts in partial fulfilment of the requirements for the degree of Doctor of Philosophy.



normal blood calcium level, and an absence of alkalosis. Rats reared on this diet usually died before any loss of weight occurred and the growth curves for deficient animals were fair but not optimum. Anorexia and inanition, according to these authors, do not play a part in this decrease in growth rate. However, when dogs were depleted of magnesium on this same diet, the nutritional failure due to the deficiency became much more apparent (3). Thus, Kruse, Orent, and McCollum concluded that magnesium deficiency manifests itself locally by hyperirritability of the nervous system and constitutionally by nutritive failure. In later papers, Kruse, McCollum, and associates have presented their findings on various phases of the biochemistry of the deficiency; namely, changes of blood composition (4), changes in composition of bone (5), and changes in the mineral metabolism (6). Recently Day, Kruse, and McCollum (7) have studied the effect of combined magnesium and calcium deficiency.

No human pathology has as yet been demonstrated to be due to magnesium deficiency. On the other hand, in animal nutrition we find the disease known as grass tetany, whose syndrome is strikingly similar to that of an experimental magnesium deficiency. Sjollem and Seekles (8, 9) have made an extensive study of this tetany and found that the first symptoms usually appear about 6 weeks after calving. The animals become nervous and restless, and develop an unsteady gait. Finally they succumb to violent clonic convulsions. The blood picture in this disease shows varying but usually reduced calcium, normal phosphorus level, and an extremely low magnesium level.

In this connection it is interesting to mention the work of Duncan, Huffman, and Robinson (10) who have produced an experimental magnesium deficiency in calves on a diet of whole milk supplemented by Fe, Cu, Mn, Si, Al, and cod liver oil. The calves on this diet underwent their first attack of tetany between the 31st and 60th day of the experiment. Most of the animals survived several attacks before they died. Their blood magnesium was consistently low, varying from 0.88 to 1.5 mg. of Mg per 100 ml. of plasma. This work is extremely interesting in that the deficiency was produced on a diet containing 10 mg. per cent of magnesium, which is about 55 times the content of the Kruse, Orent, and McCollum diet.

Many phases of the effect of magnesium deprivation have been studied in the course of the present investigation. These include the variation in the syndrome of physical changes in the experimental animals produced by varying levels of dietary magnesium and calcium and the imbalance in certain of the vitamin supplements. The alterations in chemical composition of the blood, soft tissues, and the whole animal body have been determined for the different stages of this deficiency disease. An estimate also has been gained of the minimum levels of magnesium required for normal growth, and to meet the needs of mother and young during pregnancy and lactation.

### *Experimental Procedures and Analytical Methods*

The diets were prepared from washed casein, sucrose, hydrogenated cottonseed oil,<sup>1</sup> and a purified salt mixture and vitamin supplements. Sucrose and fat were obtained free from magnesium as purchased, but it was necessary to wash the casein with acid to free it from magnesium.

For the vitamin supplements, Mead's cod liver oil was fed at a 2 per cent level as a source of vitamins A and D, and Lilly's liver extract,<sup>2</sup> and, in some cases, a repurified form of Armour's liver extract were used as a source of the vitamin G complex. Galen B, a commercial rice polishing extract, prepared according to Evans and Lepkovsky (11), was used as a source of vitamin B. In some cases this preparation was reextracted with 50 per cent alcohol. The 50 per cent alcohol extract of yeast as a source of vitamins B and G, described by Kruse, Orent, and McCollum (2), was also used for this purpose.

The salt mixtures were prepared so as to contain a minimum number of constituents in order to facilitate purification. The sources of calcium and phosphorus were the secondary and tertiary forms of calcium phosphate which were reprecipitated two or more times to free them from magnesium. The two calcium phosphates allowed the preparation of salt mixtures with varying calcium levels and relatively fixed phosphorus content. The com-

<sup>1</sup> Marketed under the trade name of Snowdrift.

<sup>2</sup> We are indebted to Vitab Products, Inc., of Berkeley and to Eli Lilly and Company for their generosity in supplying us with vitamin B and liver extracts, respectively.

ponents of the basal diets and the three different salt mixtures which have been employed are given in Table I. The magnesium contents of the various diets have been determined by the chemical analysis of the individual components and then by a check analysis of the mixed diet. The magnesium and calcium levels of the experimental diets and the nature of the water-soluble vitamin supplements supplied are recorded in Table II.

It will be seen from Table I that the magnesium level in the control diet was 50 mg. per 100 gm. of diet.

TABLE I  
*Composition of Basal Diet and Salt Mixtures*

Basal diet		Salt mixtures		
		I	II	III
	parts	gm. per 100 gm. food	gm. per 100 gm. food	gm. per 100 gm. food
Casein.....	25	NaCl..... 0.73	0.73	0.73
Fat.....	25	KCl..... 1.25	1.25	1.25
Sucrose.....	50	Fe citrate. . . . . 0.12	0.12	0.12
Cod liver oil.....	2	KI..... 0.03	0.03	0.03
Wheat germ oil.....	1	CaHPO <sub>4</sub> ..... 3.00	1.25	
		Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub> .....		3.00
		5.13	3.38	5.13

The control diets were prepared in the same manner as the deficient, except for the addition of 0.5 per cent magnesium sulfate.

Groups of healthy young rats weighing from 40 gm. up to more than 100 gm. were used as experimental animals. They were kept in a standard type screen bottom cage. A special type of food cup, consisting of a glass jar covered with an inverted wire cylinder attached to the screw top of the jar and extending to about 0.5 inch above the bottom of the jar, was used to prevent spilling. The animals were fed and watered *ad libitum*, except in the balanced feeding trials.

For the analysis of blood, tissue, and body constituents, the following analytical methods were employed. Magnesium was determined in blood by the procedure described by Greenberg and Mackey (12). In tissues, diets, and whole rat ash, a modifica-

tion of this method has been used (13). As a standard procedure on the whole rat and as a check in other instances, magnesium was precipitated as magnesium ammonium phosphate and the phosphorus was determined colorimetrically according to the procedure of Fiske and Subarow (14). Calcium was determined in the whole rat ash by titration of the oxalate with permanganate, and

TABLE II  
*Magnesium and Calcium Levels and Water-Soluble Vitamin Sources of Experimental Diets*

Diet No.*	Lot No.	Mg mg. per 100 gm.	Ca per cent	Source of vitamin B	Source of vitamin G complex
1	1	2.0	0.87	Yeast†	Yeast, Armour's liver
1	2	2.2	0.87	"	"
1	3	2.2	0.87	2% Galen B	25 mg. Lilly's liver per rat per day after depletion
1	3b	2.2	0.87	Same	50 mg. Lilly's liver per rat per day
1	3c	2.2	0.87	"	25 mg. same
1	4a, 4d	1.8	0.87	3% Galen B	2% same
2	1a, 1d	0.4	0.87	2% reextracted Galen B	1.5% Armour's liver
2	1b	0.8	0.87	Same	2% Lilly's liver
2	1c	1.2	0.87	"	Same
2	3	1.3	0.87	2% Galen B	"
2	4	1.5	0.87	3% " "	"
5-M-Ca	1-3	5.0	0.87	Same	"
5-L-Ca	1	5.0	0.39	"	"
5-H-Ca	1-4	5.0	1.16	"	"

\* The letters M, L, and H represent moderate, low, and high calcium, respectively.

† Prepared according to Kruse, Orent, and McCollum (2).

in tissues by titration with ceric sulfate according to a procedure developed by Larson (15) in this laboratory.

### Results

Profound changes are produced in the magnesium and calcium contents of the body and blood by deprivation of magnesium. The onset of these changes is very rapid, being detectable within

a few days after the animal is placed on a diet low in magnesium. The course of magnesium deficiency in the rat conforms to two fairly definite phases, the first being characterized chiefly by vasodilatation, hyperemia, and hyperexcitability, and the second being marked by nutritional failure, cachexia, and kidney damage. The transition between the two phases of the deficiency is delimited by definite changes in the trend of a number of physiological and chemical factors. The second phase of magnesium deficiency may be regarded as the time elapsing after the onset of hyperexcitability until the death of the animal. In this period growth drops off and nutritive failure becomes apparent. As the growth rate drops, other symptoms of malnutrition, such as a general loss of hair, a rough and sticky coat, ringed eye dermatitis, diarrhea, and finally edema, make their appearance.

Our observations of the outward signs of malnutrition while similar to are not identical with those made by Kruse, Orent, and McCollum. Our deficient animals did not develop erythematous desquamated areas on the skin, marked loss of hair around the eyes, on the ears, and the under surfaces of the jaws and neck, as noted by these workers; nor did the nails of our animals show an accentuated curvature and become brittle. This variation may be due to the higher magnesium level, or to other differences in the diets.

It is to be noted that for the most part we have been studying a milder condition of deficiency than the one produced by Kruse, Orent, and McCollum. In a milder form of the deficiency, the symptoms of malnutrition overshadow those of hyperexcitability, so that with diets containing 1 to 2 mg. of Mg per 100 gm. of food the second phase of the deficiency is greatly prolonged. It is in the later stages of the second phase of deprivation that the kidney pathology becomes conspicuous. The diarrhea and a ringed eye dermatitis also appear with frequency only in a deficiency of long duration.

*Growth*—Differences in growth between the control and deficient animals become significant shortly after the onset of hyperexcitability. This is one of the transition marks between the two phases of the deficiency. In the course of the present study, data have been obtained on the influence of a number of variables aside from the magnesium content of the diet on growth.

With levels up to about 2 mg. of Mg per 100 gm. of food, the effect on the growth is fairly proportional to the magnesium intake. This is shown in Fig. 1. With a diet containing about 0.4 mg. of Mg per 100 gm. of food, the stoppage of growth comes on in a very short time. With increasing magnesium content in the diet, the period of fair growth is prolonged.

The influence of the initial weights of the animals for a dietary level of about 1.5 mg. of Mg is shown in Fig. 2. It is apparent from Fig. 2 that, within limits, the period of good growth is

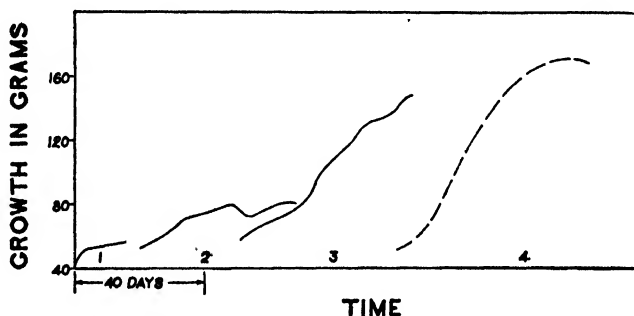


FIG. 1. Growth curves of rats reared on graduated amounts of magnesium.

Curve No.	Dietary Mg per 100 gm. food	No. of animals	Average starting weight
	mg.		gm.
1	0.4	15	43
2	1.2	10	52
3	2.2	6	58
4	50.0	5	51

practically independent of the starting weight. This period lasts about 2 to 3 weeks, and is followed by a rapid decline in the rate of growth which eventually may lead even to a loss of weight. For the first 2 to 3 weeks, the percentage weight change of the magnesium-deprived animals nearly equals or is parallel to that of the control animals of a corresponding weight. After this period the decline in the percentage weight gain is much more precipitous in the magnesium-deficient animals.

A partial explanation of the inhibition in growth on deficient

diets is to be found in Table III, which shows the daily food consumption of groups of animals on the control diet and on diets containing 1.2 and 2 mg. of Mg per 100 gm. of food. The daily food consumption of the deficient animals falls off to about two-thirds of that of the controls after the onset of excitability. Balanced feeding trials show that 70 to 75 per cent of the difference in weight gained by controls and deficient animals can be accounted for by decreased food intake. Results for two such trials are summarized in Table IV.

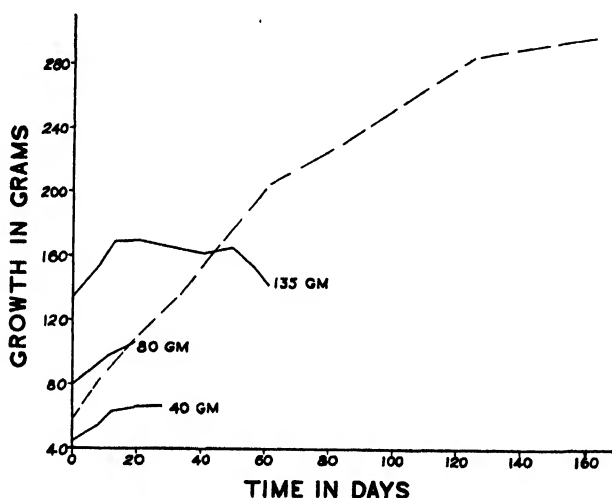


FIG. 2. Influence of the weight at the beginning of magnesium deprivation on growth. Magnesium content of the deficient diet 1.5 mg. per 100 gm. of food. The curves are drawn from the data of groups of five or more animals. Control curve represented by broken line.

In the above feeding trials the vitamin G and B levels were more than twice the level necessary for optimum growth. Thus, although the food consumption of the deficient animals was low, there was an ample amount of the B complex in the daily ration. The effect of lowering the vitamin G content of the diet is discussed in Paper II of this series (16).

*Plasma Magnesium*—The curves representing the alterations in plasma magnesium under varying degrees of magnesium deficiency are shown in Fig. 3. The analytical figures were all ob-

tained from pooled blood samples. The number of animals in a pooled sample varied between two and six. Fig. 3 represents the course of the plasma magnesium level over nearly the whole life span of groups of animals receiving less than 1 mg. (Curve 1) and 2 mg. (Curve 2) of Mg per 100 gm. of food. Curve 3 shows the results obtained over a more limited time period on animals maintained on a high calcium diet containing 5 mg. of Mg. Curve 4 of Fig. 3 represents a closer study of the changes occurring in the first phase of the deficiency in rats fed a diet containing 1.5 mg. of Mg. In this series, the influence of the starting weights of the

TABLE III  
*Relation of Growth to Food Intake in Magnesium Deprivation*

Control group. 50 mg. Mg per 100 gm. food; starting weight 100 gm.			Deficient group. 2 mg. Mg per 100 gm. food; starting weight 100 gm.			Control group. 50 mg. Mg per 100 gm. food; starting weight 65 gm.			Deficient group. 1.2 mg. Mg per 100 gm. food; starting weight 65 gm.		
No. of animals	Time	Food intake per rat per day	No. of animals	Time	Food intake per rat per day	No. of animals	Time	Food intake per rat per day	No. of animals	Time	Food intake per rat per day
	days	gm.		days	gm.		days	gm.		days	gm.
4	0-10	11.2	6	0-10	9.1	4	0-7	10.7	3	0-7	8.8
4	10-20	11.3	6	10-20	7.7	4	7-11	10.1	3	7-11	7.4
4	20-33	11.5	6	20-30	7.7	4	11-16	12.3	3	11-15	7.4
			6	30-43	7.4				1.2 mg. Mg per 100 gm. food; starting weight 50 gm.		
			5	0-10	7.4				5	0-8	6.7
			5	10-20	7.7				5	8-14	7.3
			5	20-30	7.3						

animals on the blood magnesium changes has been considered by employing three groups of rats whose average weights were 65, 80, and 100 gm., respectively. The results indicate that a considerable variation in the starting weight does not greatly affect the course of the change in the level of plasma magnesium. The points of all the weight groups fall very closely together in each time period.

Fig. 3 shows that the plasma magnesium drops at a rate which is dependent upon the degree of the deficiency of the diet. In the group reared on less than the 1 mg. of Mg diet, the plasma



magnesium reached its low level within 3 days. In the 1.5 mg of Mg series (Curve 4) it may be seen that there is a noticeable reduction in the plasma magnesium by the 3rd day of the experimental period. The minimum plasma magnesium level is not reached until 6 days have elapsed, and persists until about the 10th day. In the case of the animals on the 2 mg. of Mg diet, the plasma magnesium reached its minimum level, 0.6 mg. of Mg per 100 ml. of plasma, on about the 14th day on the diet. This low level was maintained with slight variation until shortly

TABLE IV  
*Balance Feeding Test*

No. of animals	Starting weight	Diet	Experimental period	Feeding	Food intake		Weight gain	
					Total	Per rat	Total	Per rat
	gm.		days		gm.	gm.	gm.	gm.
5	100	Control	33	<i>Ad libitum</i>	2031	406	414	83
5	100	"	33	Balanced	1141	228	229	46
5	100	Deficient	33	<i>Ad libitum</i>	1141	228	184	37

Gain of *ad libitum* control group over  
deficients..... 2.25 ratio; 125.0% difference  
Gain of balanced control group over  
deficients..... 1.24

4	50	Control	24	Balanced	638	155	219	54.7
4	50	Deficient	24	"	638	155	158	39.5

Gain of control group over deficients..... 1.32 ratio; 32.3% difference

Deficient diet = 1.8 mg. of Mg per 100 gm. of food.

after the onset of excitability, which appeared in this group about the 35th day of the experimental period.

Evidence of a back swing toward the normal level of plasma magnesium which takes place after the first reduction and shortly after the onset of hyperexcitability is afforded by each of the four experimental series shown in Fig. 3. After a second peak is reached of 2 mg. per cent of Mg or over, the plasma magnesium level falls off again slowly, but does not reach the minimum observed during the first phase of the deficiency.

The numbered arrows in Fig. 3 show that the first incidence of

convulsions in each group occurred shortly before or at the same time as the beginning of the rise in plasma magnesium. It may also be seen from the growth curves on severely deficient rats (Figs. 1 and 2) that the growth rate of these animals undergoes a sharp decrease at this same time. The development of hyperexcitability, the beginning of nutritive failure, and the beginning of a rise

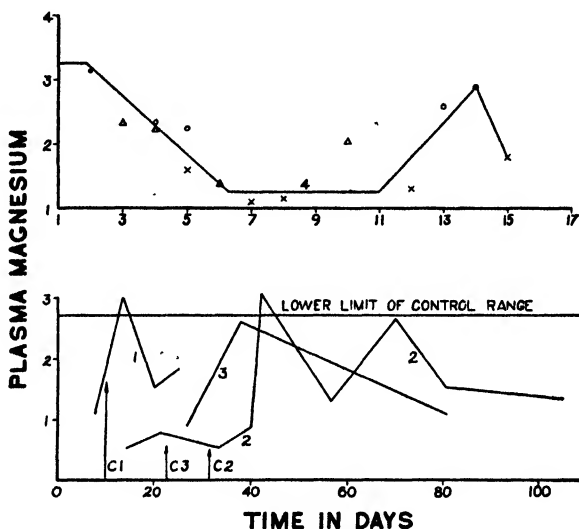


FIG. 3. Variation in the plasma magnesium with time during magnesium depletion. Analytical values are in mg. of Mg per 100 ml. on composite blood samples from two to six animals each. Curve 1, less than 1 mg. of Mg per 100 gm. of food; Curve 2, 2.0 mg. of Mg per 100 gm. of food; Curve 3, Diet 5-H-Ca. The arrows  $C_1$ ,  $C_2$ , and  $C_3$  represent the time of onset of convulsions in groups of rats whose plasma magnesium curves bear the same number. Curve 4, variation in plasma magnesium during the first phase of magnesium deficiency; magnesium content of the diet, 1.5 mg. of Mg per 100 gm. of food.  $\times$  indicates the group weighing 100 gm.;  $\Delta$  the points of the 80 gm. group; and  $O$  the points of the 65 gm. group.

in plasma magnesium level apparently occur simultaneously and mark the end of the first and the onset of the second phase of the deficiency.

It is highly interesting that the upswing of the plasma magnesium to a nearly normal level did not result in a disappearance of the hyperexcitability, so that convulsions could be induced at

this time by the hissing sound of an air blast in the same manner as at the lowest level of plasma magnesium.

*Red Corpuscle Magnesium*—A striking drop in the magnesium content of the red corpuscles takes place a little more slowly than the drop in the plasma value. Curves representing the changes in blood corpuscle magnesium are given in Fig. 4. The normal

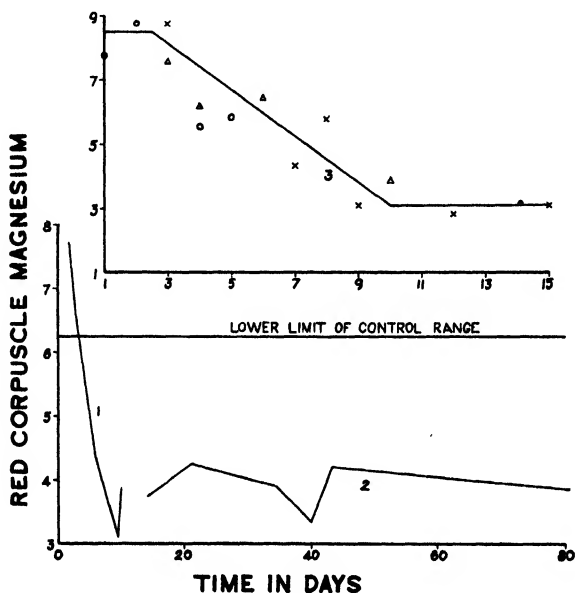


FIG. 4. Reduction of the magnesium of the red blood corpuscles of rats on magnesium-deficient diets. Analytical values are in mg. of Mg per 100 ml. of corpuscles. Curve 1, values for rats receiving less than 1.0 mg. of Mg per 100 gm. of food; Curve 2, values for rats receiving 2.0 mg. of Mg per 100 gm. of food; Curve 3, changes in red corpuscle magnesium in the first phase of magnesium deficiency, on the 1.5 mg. of Mg diet.

values for magnesium in red blood cells of rats vary from about 6.0 to 9.5 mg. of Mg per 100 ml. of cells. The corpuscle magnesium on the 1.5 mg. of Mg groups, the results of which are plotted in the upper part in Fig. 4 (Curve 3), show a drop to a value of about 3.0 mg. of Mg per 100 ml. of corpuscles within 10 days. The cells of animals on a 2 mg. of Mg diet, sacrificed from the 10th to the 40th day of the experimental period, contained 3.5

to 4.5 mg. of Mg per 100 ml. of cells, or about half the normal value. This low level of magnesium in the corpuscles is maintained or falls off slightly throughout the final stages of the deficiency.

TABLE V  
*Magnesium and Calcium Contents of Tissues*

All analytical figures are given in mg. per 100 gm. of fresh tissue.

Tissue	No. of animals	Magnesium		No. of animals	Calcium	
		Range	Mean		Range	Mean
First phase of deficiency						
Muscle.....	12	25.0-30.0	27.8	10	5.18-13.7	8.22
Kidney.....	12	16.5-24.6	21.4	10	12.2-65.8	31.5
Heart.....	8	18.3-20.0	19.3	8	8.2-13.0	10.9
Lung.....	5	11.8-15.6	13.9	5	10.0-23.2	14.5
Second phase of deficiency						
Muscle.....	38	15.2-33.5	26.5 $\pm$ 3.17*	13	7.5- 15.2	10.1
Kidney.....	36	15.6-46.7	25.2	14	14.2-232.0	106.7
Heart.....	8	20.5-26.5	23.1	9	6.1- 14.7	9.3
Brain.....	37	11.0-20.6	13.6 $\pm$ 2.17*	9	7.9- 25.5	11.7
Liver.....	27	12.3-23.6	22.2			
Skin.....	13	9.9-21.8	15.1			
Control						
Muscle.....	34	24.6-35.1	29.6 $\pm$ 0.79*	17	4.1- 7.9	5.7
Kidney.....	34	16.0-28.0	21.5	17	6.9-15.4	8.5
Heart.....	13	19.3-28.6	22.4	13	3.6- 7.9	6.4
Brain.....	22	19.8-13.2	16.6 $\pm$ 2.54*	5	6.5-15.5	10.2
Liver.....	18	18.6-25.4	22.9			
Skin.....	9	10.0-20.3	15.1			
Lung.....	6	12.0-16.0	14.3	6	8.3-18.3	14.6

\* Standard deviation.

### *Chemistry of Soft Tissues*

**Magnesium Content**—The data for the magnesium and calcium contents of the soft tissues in the first and second phases of the deficiency are summarized in Table V. No striking changes from normal have been noted in the magnesium content of the soft

tissues at any time during the progress of the deficiency. In muscle the mean for deficient animals falls 10 per cent below that for the control group, and in brain 20 per cent below that for the control.

In the case of muscle, the standard deviation for control figures is 0.79 and for deficient animals 3.17. According to the formula  $\sigma M = \sigma x / \sqrt{n}$ , where  $\sigma M$  = standard deviation of the mean and  $\sigma x$  = standard deviation of the group, the standard deviation of the mean is 1.35 in the case of deficient animals, and 0.51 in the case of the controls. Then, according to the equation,

$$\sigma(M_1 - M_2) = \sqrt{\sigma(M_1)^2 + \sigma(M_2)^2}$$

the standard deviation of the mean difference is 0.555. Since this value is less than one-third of 3.1, the differences in the means, we may consider the variation to be significant. In the case of brain the standard deviation of the mean difference also is less than one-third of the difference in mean between the control and deficient groups. These facts strengthen the theory of depletion from these tissues. In the other soft tissues observed, the values of the means of deficient groups fall very close to the means for the corresponding controls, from which it would appear that there is no depletion in heart, lung, skin, liver, or kidney.

*Calcium Content*—During the first phase of the deficiency there is a rise in the calcium content of heart, muscle, and kidney. The average increase amounts to 80 per cent in heart, 40 per cent in muscle, and 300 per cent in kidney. During the second phase of the deficiency the calcium content of muscle and kidney continues to rise, while the high level reached in heart during the first phase is maintained during this period. The calcium content in the muscle finally reached about 2-fold the normal value, while in the kidneys the average figure is 12 times the mean figure for control kidneys, and as much as 26-fold increase in kidney calcium has been observed. In those kidneys in which extremely high values for calcium were observed, the magnesium content was also high, showing an accumulation of as much as twice the normal amount of magnesium for each 100 gm. of tissue.

*Water Content*—The water content of the soft tissues of deficient animals showed no significant alteration from that of the controls.

*Mineral Content of Whole Body*

The changes in body calcium and magnesium for the first phase of the deficiency are shown in Fig. 5, and for the second phase in Fig. 6. The decrease in the percentage of the total body magnesium parallels the decreased values for plasma and red cells and reaches a low level before the onset of the first convulsions. The rate at which this value falls off is roughly proportional to the

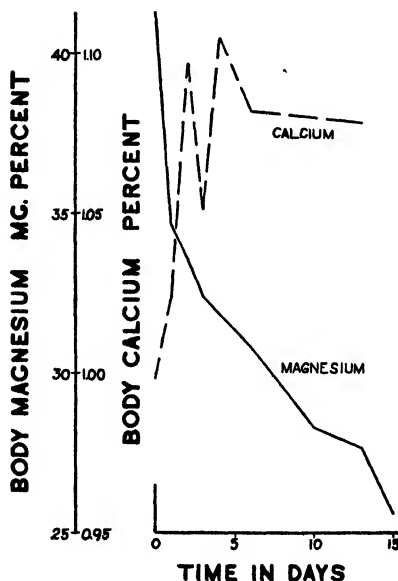


FIG. 5. Variation in whole body magnesium and calcium in the first phase of magnesium deficiency. Magnesium content of the diet 1.5 mg. of Mg per 100 gm. of food. Starting weights of animals between 60 and 80 gm. Data for magnesium expressed in mg. and for calcium in gm. per 100 gm. of fresh carcass.

severity of the deficiency. The minimum level reached in the first phase of the deficiency (Fig. 5) is from 22 to 25 mg. of Mg per 100 gm. of rat or about two-thirds the normal level. During the second phase of the deficiency, the per cent body magnesium falls off only slightly from the low level reached previously. Values below 20 mg. of Mg per 100 gm. of rat are observed only rarely, although the extremely low percentage of 13.9 mg. of Mg per 100

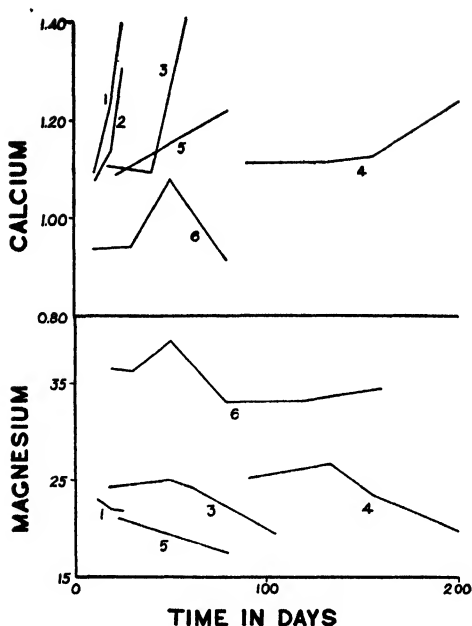


FIG. 6. Variation in the calcium and magnesium content of the whole rat carcass with time during magnesium deprivation. Data for magnesium are expressed in mg. and for calcium in gm. per 100 gm. of fresh carcass. Each numbered curve represents data on the same group of animals for both calcium and magnesium. The magnesium values of the second series are omitted from the figure because they overlapped Curve 1. The dietary levels of magnesium represented by the curves are:

Curve No.	Starting weight	Dietary Mg per 100 gm. food
	gm.	mg.
1	40	0.4
2	50-60	1.2
3	50-60	2.0
4	50-60	5.0
5	50-60	5-H-Ca
6	50-60	Control

gm. has been obtained. The absolute amounts of magnesium per animal present in rats after depletion are reported in Table VI. It will be noted that there is seldom an actual loss of magnesium from the organism.

The calcium percentage of the whole body, on the other hand, increases throughout the deficiency. From Fig. 5 it is apparent that the increase in the per cent calcium sets in within a few days after the beginning of magnesium deprivation. On a diet containing 0.4 mg. of Mg the rise in per cent of body calcium sets in about the 12th day and reaches a value 40 per cent above the normal mean by the 20th day on the experimental diet. On a 2 mg. of Mg diet the rise is much slower and reaches a value of 1.50 per cent calcium only after 50 days. On a 5 mg. of Mg diet a still slower rise is observed.

The change in the per cent of body calcium mainly represents a relative rather than an actual increase in the total store of body

TABLE VI  
*Change in Total Body Magnesium during Depletion*

Mg in diet per 100 gm. food	No. of animals	Starting weight of animals	Time on diet	Calculated initial body Mg per animal	Body Mg after depletion, per animal	
					Range	Mean
mg.		gm.	days	mg.	mg.	mg.
2.0	23	40- 60	40-105	14.3	17.2-43.0	29.9
2.0	8	40- 60	40- 70	14.3	17.2-41.5	24.6
2.0	6	40- 60	105	14.3	25.7-39.0	31.2
2.0	9	90-110	0- 40	40.0	26.6-43.0	33.7
1.2	3	40- 50	12- 25	14.1	13.4-14.8	13.9
1.2	13	50- 60	12- 25	14.3	13.3-19.6	16.7
0.4	11	30- 40	12- 25	13.9	9.3-15.3	11.6

calcium. This is demonstrated by the comparison between the changes in total body calcium of control and magnesium-deprived animals given in Table VII. In the first phase of the deficiency there is a tendency toward a greater retention of calcium in the magnesium-deprived animals than in the control animals. This is supported by the greater increase in the total body calcium of rats whose depletion was started at 35 and at 42 days of age. However, in the second phase of the deficiency, probably because of decreased food consumption, the calcium retention of the deficient animals is less than that of the controls, as may be seen in the column in Table VII giving the total body calcium of rats placed on the low magnesium diet at 28 days of age.



TABLE VII  
Change in Total Body Calcium Content during Magnesium Deprivation

Age of animals	No. of animals	Controls		No. of animals	Depletion started at 28 days		No. of animals	Depletion started at 33 days*		No. of animals	Depletion started at 42 days†	
		Range	Mean gm.		Range	Mean gm.		Range	Mean gm.		Range	Mean gm.
30-40	2	0.75-0.90	0.80	8	0.58-0.79	0.68*	3	0.80-1.10	0.90	3	0.95-1.10	1.00
40-50	6	0.70-1.10	0.90	10	0.63-0.80	0.70*	4	0.95-1.10	1.00	5	1.30-2.00	1.50
50-60	3	0.80-1.20	1.00	5	0.72-0.96	0.77*	4	1.05-1.35	1.20	2	1.35-1.75	1.50
60-70	3	1.40-1.60	1.50	2	0.90-1.00	0.95*				4		
70-80	4	1.30-1.65	1.55	2	1.10-1.50	1.35†						
80-90	4	1.25-1.65	1.50	3	1.10-1.45	1.25†						
90-100	2	1.90-2.10	2.00	2	1.30-1.40	1.35†						

\* The diet contained 1.2 mg. of Mg per 100 gm. of food.

† The diet contained 2 mg. of Mg per 100 gm. of food.

The increase in the percentage of body calcium appears to result from the progressive emaciation which ensues. This leads to an increase in the proportion of the skeleton to the total body weight and consequently to a relative increase in the body calcium.

#### DISCUSSION

In general, we have been able to confirm Kruse, Orent, and McCollum's (2) findings concerning the nature of the syndrome of severe magnesium deficiency. However, we find that malnutrition plays a very important part in the later stages of a severe deficiency and, as the level of magnesium is raised, the symptoms of malnutrition overshadow those of hyperexcitability. Thus the symptoms observed with a magnesium level of 1.5 to 2.0 mg. of Mg per 100 gm. of food resemble more closely those reported by Orent, Kruse, and McCollum (5) for dogs than those reported for rats. Furthermore, we find that there is a considerable decrease in food intake in deficient animals and that this loss of appetite accounts for 70 to 75 per cent of the difference between the weight gained by the deficient and control animals. We must then conclude that the effect of magnesium deficiency is additive with the effect of calcium deficiency in producing the marked inanition and nutritive failure in rats observed by Day, Kruse, and McCollum (7) on a diet deficient in both calcium and magnesium.

The results reported in Fig. 3 seem to warrant the conclusion that plasma magnesium reaches a minimum value shortly after the start of the deficiency, moves back to an only slightly sub-normal value shortly after the appearance of hyperexcitability, and then falls off again but never again to such low levels as those observed in the first phase of the deficiency. The minimum levels never fall below 1.0 mg. per cent of plasma in this latter phase. It is of interest that the hyperirritability persists even during the upswing to nearly normal plasma magnesium levels. Evidently the reduction in the level of the plasma magnesium is not directly responsible for the condition of hyperirritability.

The rapid drop which is observed in blood magnesium may be attributed to the fact that animals in the first stage of the deficiency have always been young and actively growing, so that there are tremendous demands for magnesium for skeletal syn-

thesis on the one hand, and for the production of increased amounts of the soft tissues on the other. As long as growth continues at a high rate of speed, the soft tissues and bone incorporate into themselves any available magnesium, and the amount left to circulate in the blood stream is kept low.

The subsequent temporary rise in plasma Mg level might be explained by one of the simultaneous effects of the following processes: a diminution in the rate of magnesium excretion, a drop in the absolute amount which is deposited in bone during this period, or the marked reduction which occurs in the amount of magnesium in the corpuscles.

The manner in which magnesium is reduced in the corpuscles is open to speculation. It is a generally accepted theory that cations, with the exception of hydrogen ion, do not cross the red cell membrane. If this is true, we must assume that the depletion takes place through the production of red cells poor in magnesium. However, *in vitro* experiments in this laboratory show that the magnesium may increase in the corpuscles to about 140 per cent of its original value upon the addition of magnesium sulfate to give levels 50-fold the normal magnesium concentration of blood. The difference in these experiments cannot be accounted for by changes in volume of the cells. Thus it is perhaps possible that under certain circumstances magnesium can diffuse across the red cell membrane, although only very slowly, and this mechanism comes into play on the depletion of the corpuscles. The final falling off in the magnesium content of the plasma can be explained by the continued excretion of appreciable amounts of magnesium, and the steady accumulation of some magnesium in the bones in the later stages of deprivation (see (5, 6)).

Our observations on the increase in the per cent of calcium in the whole carcasses of rats are in good agreement with those of Medes (17) who found an increase in calcium in the whole body with a diet moderately low in magnesium. It has already been pointed out that this actually represents an increased retention of body calcium only in the first phase of the deficiency, but only a relative increase in the second phase owing to an increase in the ratio of the skeleton to the total body weight. It is of interest in this connection that Orent, Kruse, and McCollum (3) have observed that an increased rate of deposition of this element in bone is

confined to the first phase of the deficiency. Thus they found that the curve for the rate of deposition of calcium in the bones of the deficient animals early reached a level higher than that of the controls and then ran parallel to the control curve in the later stages of the deficiency.

The decrease in per cent magnesium content of the whole carcass cannot be interpreted as an actual loss of magnesium to the body except in diets containing as little as 0.4 to 1.2 mg. of Mg per 100 gm. of food. Even in such a severe deficiency the loss is slight, as shown by the fact that the average total body content for rats depleted on these diets is 12.2 mg. of Mg, while the total body figure for controls of the age at which these animals were placed on the diet is 14.0 mg. of Mg (see Table VI). The decrease in the per cent of body magnesium on diets containing 1.5 to 5.0 mg. of Mg per 100 gm. of diet usually results from a tremendously reduced rate of accumulation, and not because of an actual loss of magnesium. Apparently the animals hold on tenaciously to their acquired store of magnesium, but can accumulate little or no extra magnesium from these diets.

#### SUMMARY

1. The study of magnesium deprivation has revealed that there are two phases to the deficiency. The first phase is manifested chiefly by vasodilatation, hyperemia, and hyperexcitability. The second phase is marked by the development of malnutrition, cachexia, and kidney damage.

2. In the first phase, the plasma magnesium level undergoes a sharp drop and then rises to a peak shortly after the onset of hyperexcitability. It falls off again during the second phase, at a relatively slow rate, to reach values not lower than 1.0 mg. per cent of plasma magnesium.

3. The magnesium content of the red corpuscles is reduced to about half the normal amount during the early phase of depletion and remains fairly constant after this low level is reached.

4. There is little retardation of growth in the first phase, and a marked reduction in growth in the second phase of magnesium deficiency.

5. During depletion there is no change in water content of the soft tissues. No striking changes are noted in the magnesium

contents of the soft tissues, but the results indicate a slight withdrawal of magnesium from brain and muscle. Calcium is increased in heart and muscle from 50 to 100 per cent; in kidney as much as 15-fold.

6. The percentage of body magnesium is reduced to about two-thirds the normal level and the per cent of body calcium is increased to  $1\frac{1}{2}$  times the normal value by prolonged depletion. These changes are mainly relative. The total magnesium content of the body is usually not decreased and, except in the first phase of the deficiency, there is less total calcium content in the bodies of the deficient animals than in controls of the same age.

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## THE BIOCHEMISTRY OF MAGNESIUM DEFICIENCY\*

### II. THE MINIMUM MAGNESIUM REQUIREMENT FOR GROWTH, GESTATION, AND LACTATION, AND THE EFFECT OF THE DIETARY CALCIUM LEVEL THEREON

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Information concerning the minimum intake which will maintain the well being of the animal is an important item in the evaluation of the nutritional significance of a dietary component. As will be brought out below, a fixed value cannot be given for the minimum requirement of a given element. The amount required will be found to vary with varying conditions of the life history of the animal, and also in relation to the intake of other components of the diet.

To obtain an estimate of the minimum magnesium requirement for the rat, we have attempted to determine the lowest magnesium level in the diet at which animals grow and reproduce normally and are maintained in a state of good health otherwise. In connection with this work it was found that the dietary calcium level has a very important effect in altering the minimum magnesium requirement.

Certain data were already available which gave a clue to the amount of magnesium necessary to meet the needs of the rat dur-

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† This communication is constructed from a thesis submitted to the Graduate Division of the University of California by Elma V. Tufts in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

ing growth. Osborne and Mendel (1) found that rats on a dietary level of 12 mg. of Mg per 100 gm. of food grew normally and showed no other ill effects for a period of nearly a year. Subsequently they dropped off about 20 per cent in weight. Medes (2) obtained good growth and observed no external ill effects on a diet containing 6 mg. of Mg per 100 gm. of food. Chemically, however, she did find a reduction in the percentage of magnesium and some increase in the calcium percentage of the whole rat carcass. It is of interest to note that the calcium contents of the above diets were fairly low. The Osborne and Mendel diet contained about 0.2 per cent calcium, and in the work of Medes it varied between 0.2 and 0.4 per cent. No data have appeared hitherto which would give a clue as to the amount of magnesium in the diet required to meet the needs of the animal during the period of gestation and lactation.

### *Diets and Experimental Procedures*

The general composition of the diets employed in this work, as well as the analytical methods employed, are the same as those given in Paper I of this series (3). The basal ration consisted of casein, sucrose, and fat supplemented with the fat-soluble and the water-soluble vitamins. For sources of the water-soluble vitamins, a 3 per cent level of Galen B was used to supply vitamin B, and a 2 per cent Lilly's liver extract<sup>1</sup> to supply the vitamin G complex. The magnesium, calcium, and phosphorus composition of the diets is recorded in Table I. In all cases control experiments were run with diets which differed from the deficient series only by the addition of magnesium sulfate to give a content of 50 mg. of Mg per 100 gm. of food.

### *Results*

*With Diets of Moderate Calcium Content*—The work of Medes indicated that a diet containing 6 mg. of Mg per 100 gm. of food was approaching the minimum requirement of this element. With this as a guide, the dietary level of 5 mg. of Mg per 100 gm. of food was chosen for use in the main part of the present study.

<sup>1</sup> We are indebted to Vitab Products, Inc., of Berkeley and to Eli Lilly and Company for their generosity in supplying us with vitamin B and liver extracts, respectively.

It has been pointed out in another place (4) that the symptoms of magnesium deficiency vary more and more in their time of onset and their time relationship to each other as the magnesium in the diet is increased. At a level of 2 mg. of Mg per 100 gm. of food the deficiency appears as a fairly fixed syndrome of vasodilatation, followed by hyperexcitability and malnutrition. At the 5 mg. of magnesium level the symptoms of vasodilatation and hyperexcitability may develop, but the picture is extremely variable. Vasodilatation may develop to an extreme degree and is recurrent. Only about one-fourth of the animals became hyperexcitable during the first 2 or 3 weeks of the experiment, and this symptom soon disappeared. Other manifestations of hyperexcitability did not appear until after more than 70 days on the diet.

TABLE I  
*Mineral Composition of Experimental Diets*

Diet No.	Magnesium per 100 gm. food	Calcium	Phosphorus
	<i>mg.</i>	<i>per cent</i>	<i>per cent</i>
5-L-Ca	5.0	0.39	0.45
5-M-Ca	5.0	0.87	0.80
5-H-Ca	5.0	1.16	0.75
13-H-Ca	13.0	1.66	1.00

In diets labeled L and M, calcium was supplied as  $\text{CaHPO}_4$ ; in diets H, as  $\text{Ca}_3(\text{PO}_4)_2$ .

Rats which had no previous record of convulsions frequently became hyperexcitable when subjected to the added strain of pregnancy and lactation.

With a low or moderate calcium content, 5 mg. of magnesium are adequate for normal growth. As is shown in Fig. 1 (Curve 2), the growth on this diet almost exactly parallels that for the controls (Curve 3). The rats on this regimen showed no tendency to fall off in weight even after more than 150 days on the diet. Moreover, they showed no other external signs of malnutrition such as rough coats, loss of hair, dermatitis, or edema, but remained in a sleek, well kept condition throughout the experimental period.

It was possible to breed successfully the rats reared on this diet.



The animals were bred at an age of 80 to 120 days and gave birth to normal young with an average weight of 5 gm. In a great many cases the mothers refused to nurse the young and either let them starve or devoured them. We succeeded, however, in rearing a number of litters of four young rats each to a month or more of age. Thus when the calcium content is not over about 0.9 per cent, the level of 5 mg. of Mg per 100 gm. of food appears to be on the border line of the dietary requirement. At this magnesium content, symptoms of deficiency appear only at times of great stress in the metabolic processes; namely, during the most active period of growth, and during pregnancy and lactation.

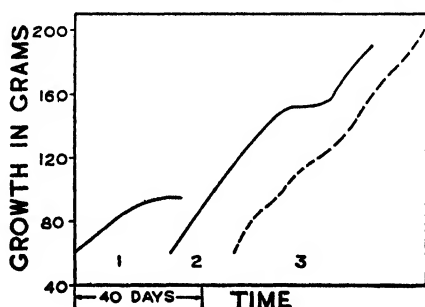


FIG. 1. Effect of low and high calcium diets on growth in magnesium deficiency. The curves are drawn from the data of groups of six animals each. Curve 1, Diet 5-H-Ca; Curve 2, Diet 5-L-Ca; Curve 3, control diet. The control curve is represented by the broken line.

The young from the mothers on this diet are normal in weight and in magnesium content at birth, but develop a deficiency of body magnesium during lactation. The per cent magnesium did not increase in their bodies as it does in young rats suckled by mothers on a diet higher in magnesium. The deficiency in body magnesium is accompanied by the usual symptoms of vasodilatation and hyperexcitability. On the other hand, the growth curves and external appearance of the young are normal over the lactation period. The kidneys of these young rats sacrificed at weaning showed no evidence of renal damage.

Since the hyperirritability and deficiency of body magnesium develop during the nursing period, we may conclude that the mother's milk was deficient in magnesium.

It is interesting to note at this point that the young whose mothers were on the control diet containing 50 mg. of Mg per 100 gm. of food were somewhat lower in total magnesium content than were the stock animals of the same age. Evidently even the control diet is not optimum in magnesium content for lactation.

The magnesium contents of the bodies of deficient, control, and stock young are summarized in Table II.

*Effect of Raising Calcium Content of Diet*—By increasing the calcium content from 0.87 to 1.16 and 1.66 per cent in the diet we have been able to show the remarkable influence of the calcium intake on magnesium deficiency.

TABLE II

*Comparison of Body Magnesium Content of Young Rats from Litters of Mothers on Experimental Diets and Those on Stock Diet*

Age	No. of animals	Whole body magnesium, deficient animals		No. of animals	Whole body magnesium, controls		No. of animals	Whole body magnesium, stock animals	
		Range	Mean		Range	Mean		Range	Mean
wks.		mg. per cent	mg. per cent		mg. per cent	mg. per cent		mg. per cent	mg. per cent
0	9	14.4-24.4	19.8				12	21.0-22.9	21.7
3	6	18.8-23.3	20.0	3	24.6-26.0	25.5	8	33.5-44.5	36.9
5	2		17.4	4	26.2-35.4	29.9	5	36.7-44.2	39.2
6	2		18.8				4	37.4-40.7	39.3

A diet high in calcium shortens the life span and increases the incidence and severity of the convulsive attacks of magnesium-deficient animals. It also diminishes the time before the onset and increases the intensity of vasodilatation. With a 1.16 per cent calcium content, the 5 mg. of magnesium diet is no longer on the border line of sufficiency. Thus the performance of the experimental animals on the Diet 5-H-Ca is comparable in every respect to that of animals fed a diet containing 1.5 to 2.0 mg. of Mg per 100 gm. of food. The poor growth performance obtained on this diet is shown in Fig. 1 (Curve 1).

The minimal magnesium requirement for normal growth on diets with a calcium content of 1.66 per cent is approached only

when the magnesium level is raised to 13 mg. per 100 gm. of food. On Diet 13-H-Ca, the growth of the male rats kept close pace with the growth of the controls. The growth curve of the females, however, while fairly good, was still significantly below that of the control animals. The average growth curve of the females on this diet is plotted in Fig. 2.

While the rats on the 13 mg. of magnesium diet remained sleek and in a well kept condition as far as their coats were concerned, other symptoms of magnesium deficiency showed up in some of the animals. Out of six females, convulsive seizures could be induced in two, and three of them developed an edema toward the end of the experimental period.

Aside from any visible signs of hyperirritability and malnutrition, an examination of the chemical composition of the blood,

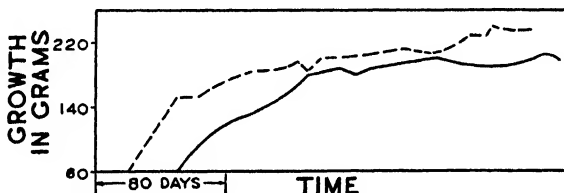


FIG. 2. Growth curves of female rats on Diet 13-H-Ca. Control (broken line) data from five rats; deficient (full line) data from six rats.

tissues, and whole body makes it evident that some degree of magnesium deficiency develops in the animal with every one of the high calcium diets listed in Table I, and even with the border line diet of 5 mg. of magnesium, containing 0.87 per cent calcium. The analytical data which support this conclusion are summarized in Tables III and IV. The magnesium content of both the blood plasma and the red corpuscles is greatly reduced on these diets. It may be seen from Table III that the plasma magnesium is lowered to about a third or less of the control level, and the red corpuscle magnesium to about half the control level.

Examination of the whole body contents, the results of which are reported in Table IV, shows that a considerable reduction has taken place in the magnesium and an increase in the per cent of calcium in the carcass. Except on Diet 13-H-Ca, the extent of the calcium increase is less than was found in rats reared on diets

more deficient in magnesium. The loss of body magnesium, however, is very marked, particularly on Diet 5-H-Ca. The lowest values of body magnesium found in the condition of magnesium

TABLE III  
*Blood Magnesium (in Mg. Per Cent)*

Diet No.	Days	Plasma Mg		Red corpuscle Mg	
		Deficient animals	Controls	Deficient animals	Controls
5-M-Ca	77	1.48	3.75		
5-M-Ca	133	1.38	3.10		
5-M-Ca	153	1.42	3.30	3.30	6.25
5-M-Ca	156	1.69	3.65	2.65	6.60
5-H-Ca	27	0.90		5.35	
5-H-Ca	37	2.13	3.75	3.75	
5-H-Ca	81	1.10	3.80		
13-H-Ca	127	1.65		4.65	
13-H-Ca	139	1.15	2.10	2.50	6.20
13-H-Ca	210	1.20	3.05	5.35	7.40

TABLE IV  
*Magnesium and Calcium Content of Whole Rats*

Diet No.	Dietary Mg per 100 gm. food	Dietary Ca	No. of animals	Time on diet	Body Mg		Body Ca, mean
					Range	Mean	
	mg.	per cent		days	mg. per cent	mg. per cent	per cent
Control	50	0.87	2	140-180	33.2-36.2	34.7	0.91
"	50	1.66	2	260	33.4-39.9	36.6	1.79
5-M-Ca	5.0	0.87	4	91	21.3-28.9	25.3	1.12
5-M-Ca	5.0	0.87	4	133	21.9-34.3	26.7	1.12
5-L-Ca	5.0	0.39	3	155	18.0-25.4	23.7	1.13
5-L-Ca	5.0	0.39	1	200		19.7	1.24
5-H-Ca	5.0	1.16	3	23	15.5-23.6	21.7	1.09
5-H-Ca	5.0	1.16	4	81	13.0-20.6	17.6	1.22
13-H-Ca	13.0	1.66	1	121		24.3	2.00
13-H-Ca	13.0	1.66	2	139	21.8-22.5	22.2	2.36
13-H-Ca	13.0	1.66	2	260		21.7	2.30

deficiency developed on this diet. After 81 days on the diet, the average body magnesium content was 17.6 mg. per cent, with a low of 13.9 per cent.

The magnesium content of such important viscera as heart, kidney, and muscle is not materially affected by these diets. However, no large reduction in tissue magnesium has been found even with the severest degree of magnesium deficiency (3). As was found in the case of the diets lower in magnesium, there is an increase in the calcium content of muscle, heart, and kidney even on the diets that border on the point of meeting the minimal magnesium requirement of the animals. The increase in the calcium content of the heart and muscle, on the average, is somewhere between 50 and 100 per cent. The increment of calcium in the kidney is very much higher. This, of course, is associated with the development of renal lesions such as have been described in another communication (5). After the animals had been between 210 and 260 days on Diet 13-H-Ca, the calcium in the kidney reached the enormous average value of 235 mg. per cent of fresh kidney, with a high of 340 mg. per cent. It is interesting to note that, while the kidneys of the control animals on this diet also manifested signs of kidney degeneration, although of a much milder degree, they showed no evidence of an increasing calcium content.

The reproductive performance on the high calcium diets was extremely poor. This was true of both the control and deficient animals. On Diet 5-H-Ca, few of the animals survived long enough, and those that did were in too poor a condition to breed. In the group on Diet 13-H-Ca, positive evidence of pregnancy, such as the erythrocyte sign, was elicited in almost 100 per cent of the control and deficient females. However, in all cases the litters were either resorbed, or, if born, were eaten by the mothers immediately after birth.

*Effect of Vitamin G Complex*—Investigation of the vitamin G complex as a possible factor in the picture of magnesium deficiency was undertaken because it was observed that both the control and deficient animals showed signs of malnutrition when the water-soluble vitamin supplement was given in the form of an alcoholic extract of yeast, prepared according to the directions of Kruse, Orent, and McCollum (6) from yeast powder purchased from the Northwestern Yeast Company.

It has been pointed out elsewhere (4) that a high intake of vitamin G apparently lengthens the time elapsing before symptoms of hyperexcitability appear, and lessens the severity of the symp-

toms. The influence of vitamin G on growth in relation to magnesium deficiency is shown in Fig. 3. As Curves 1 and 2 show,

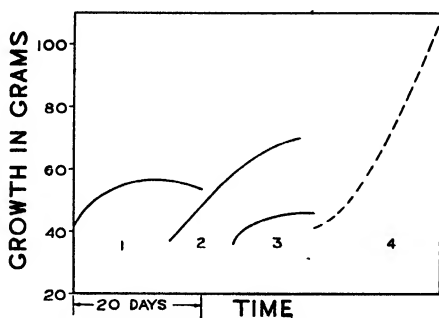


FIG. 3. Influence of the vitamin G content of the diet on growth in magnesium deficiency. Magnesium content of the deficient diet, about 0.8 mg. of Mg per 100 gm. of food. The curves are drawn from the data of groups of six animals each. Curve 1, low vitamin G, Mg-deficient; curve 2, low vitamin G, control. Supplement, 0.15 ml. per rat per day of Armour's liver extract. Curve 3, high vitamin G, Mg-deficient; Curve 4 (broken line), high vitamin G, control. Supplement, 2 per cent Lilly's liver extract.

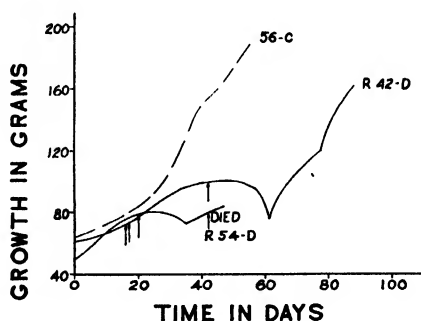


FIG. 4. Growth curves during magnesium depletion and subsequent recovery from the deficiency. The arrows represent times of injection of 1 mg. of Mg intraperitoneally. Rat 54-D received the deficient diet, containing 1.2 mg. of Mg per 100 gm. of food. Rat 42-D, fed the above diet for 61 days. It received injections of 5 mg. of crystalline riboflavin daily from the 50th to the 57th day. It was transferred to the control diet from the 61st day until the end of the experimental period. Rat 56-C (broken line) received the control diet.

when the vitamin G level of the diet is low (allowing control growth of 8 to 14 gm. per week), the difference in growth between

control and deficient animals is obscured. When the vitamin G content is ample, the differences become marked (Curves 3 and 4).

With a diet containing 1.5 mg. of Mg per 100 gm. of food or less, the symptoms of malnutrition cannot be prevented by high intake of extracts of the vitamin G and B complex, nor can they be alleviated by the injection of extra amounts of flavin. This is aptly shown by the case of Rat 42-D whose experimental course is illustrated in Figs. 4 and 5. As is shown in Fig. 4, administration of large amounts of vitamin G did not change its downward course

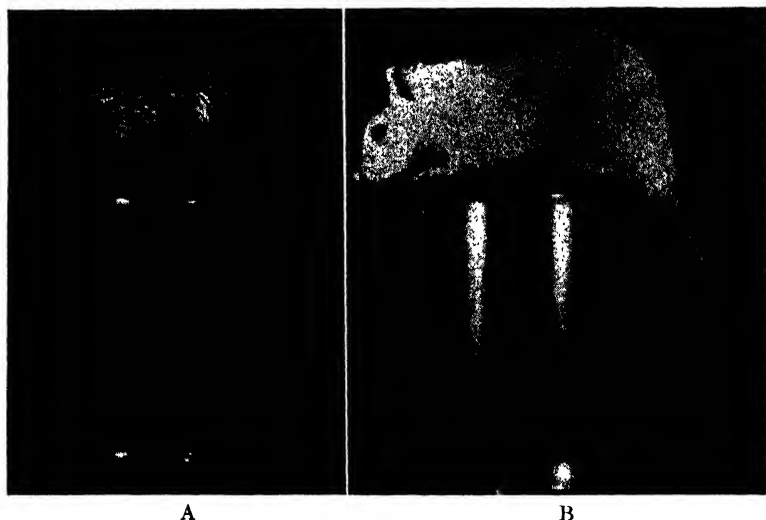


FIG. 5. *A*, photograph of a rat (No. 42-D) after 54 days on a magnesium-deficient diet (1.2 mg. of Mg per 100 gm. of food). *B*, the same rat at 91 days, after being transferred to the control diet on the 61st day.

in the least. Addition of magnesium to the diet of this animal affected an increase in growth and disappearance of all external signs of malnutrition. Thus the vitamin G complex seems to have no *direct* relationship to the state of malnutrition developed as a consequence of magnesium deprivation.

#### DISCUSSION

The present work indicates that under otherwise optimum conditions, a magnesium intake in the diet of approximately 4 mg.

per kilo of body weight per day approaches the minimal magnesium requirement of the rat.

In man Wang, Kaucher, and Wing (7) found that there was almost no net retention of magnesium in adolescent girls on a magnesium intake varying between 6.0 and 10.2 mg. per kilo of body weight. The results of these experiments have been summarized by the authors as follows: On twenty-two girls of an average age of 13 years, on an average intake of 8 mg. of Mg per kilo of body weight, the average total retention per 24 hours was 17 mg. of magnesium, or 0.4 mg. per kilo of body weight. •

A magnesium balance was obtained by Tibbetts and Aub (8) with hospital patients on an intake of about 4 mg. per kilo of body weight per day, and magnesium storage in active subjects when the intake was about 6 mg. per kilo of body weight per day.

The relation of the calcium content of the body to the dietary calcium level is of interest in connection with the present work. Sherman and coworkers (9) have shown that there is an increase in the percentage of body calcium with increasing amounts of calcium in the diet. Whitcher, Booher, and Sherman (10) found that when the allowance of dietary calcium reaches a liberal level, there is a considerable range over which a calcium increase has no appreciable effect on the amount of calcium which is accumulated. This range is between 0.5 and 0.8 per cent calcium.

The percentage of calcium in the carcasses of our control animals compares well with that found by the above authors at the same levels of dietary calcium. In our diet containing 1.66 per cent calcium, which is far above the amount used by Sherman and coworkers, the body calcium is increased to exceedingly high values. In the controls it attained the high value of 1.79 per cent. In the animals on Diet 13-H-Ca it reached the enormous figure of 2.30 per cent after 210 to 260 days on the diet. It is of interest to note that the animals on low magnesium diets show an accumulation of calcium in heart, muscle, and, particularly, of course, in the kidneys, while the control animals on the 1.66 per cent calcium diet show no accumulation in these viscera.

#### SUMMARY

1. With diets of liberal normal calcium content, and an optimum content of the vitamin G and B complex, the magnesium



level of 5 mg. per 100 gm. of food was found to be the border line amount necessary for good growth.

2. Females on this diet gave birth to young of normal weight and normal magnesium content. These young rats while suckling, however, developed all the symptoms of deficiency at the age of 2 or 3 weeks. Furthermore, the per cent magnesium did not increase in their bodies as it does in young rats suckled by mothers on a normal diet. Since the syndrome developed during the nursing period, we may conclude that the mother's milk was deficient in magnesium.

3. A high content of calcium in the diet increases the severity of magnesium deficiency and raises the amount necessary to meet the minimal magnesium requirements.

4. The minimal magnesium requirement is raised to some extent by diminishing the vitamin G intake to less than 6 Chase-Sherman units per day. However, the vitamin G complex bears no direct relation to the state of malnutrition which develops as a consequence of magnesium deficiency.

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# THE EFFECT OF GLYCINE UPON THE ACTIVITY COEFFICIENT OF GLYCINE, EGG ALBUMIN, AND CARBOXYHEMOGLOBIN \*

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The interaction of amino acids and proteins with salts has been studied fairly extensively (1, 2), but relatively little work has been done on the effect of zwitter ions upon themselves or other compounds of the same kind. Freezing point measurements have been made on solutions of some amino acids (3, 4) and the effect of a few amino acids on the solubility of cystine has been reported briefly (1). In this paper the effect of glycine on its own activity coefficient and on those of two proteins, egg albumin and carboxy-hemoglobin, has been studied.

## *Preparation of Materials*

*Glycine*—The glycine was a commercial preparation, recrystallized three times by dissolving in water and precipitating with an equal volume of ethyl alcohol. N = 18.63 per cent; theoretical = 18.67 per cent.

*Potassium Chloride*—The KCl was recrystallized three times from hot water and dried by fusing in a platinum crucible.

*Egg Albumin*—Crystalline egg albumin was prepared according to the method of Sørensen (5). After the third recrystallization the salt was removed and the crystals at the same time brought into solution by dialysis against running tap water in collodion bags, followed by electrodialysis against distilled water.

*Carboxyhemoglobin*—Considerable difficulty was encountered

\* This report is from a dissertation submitted by the author in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

in attempting to prepare hemoglobin crystals with even approximately reproducible solubility in water. Therefore, several methods of preparation were tried. In each case the starting material was fresh horse blood cells<sup>1</sup> washed four times by centrifugation with 1 per cent NaCl, and hemolyzed by evacuating and shaking with toluene. The stromata were removed after the first crystallization by shaking with toluene. On centrifuging, the stromata were held at the toluene layer. The first two lots of cells came from oxalated blood, but for the third pyrophosphate had been used as anticoagulant.

Three methods of crystallization were used. Whenever possible the solutions or suspensions were kept immersed in ice water when not in the ice box. In each case before crystallization the hemoglobin solution, after evacuation to remove oxygen, was saturated with CO; after crystallization the mixture was stored in the ice box under CO and toluene.

*Method 1. Precipitation with HCl*—The method of Ferry and Green (6, 7) was used with slight modifications: 0.2 N HCl was used as precipitating agent in place of 0.1 N HCl, and was added until the pH of the solution was between 6.7 and 6.8.<sup>2</sup> The final crystals were dialyzed for 2 days in the ice box against distilled water.

*Method 2. Precipitation with CO<sub>2</sub>*—The method of Heidelberger (10) was followed except that after the first crystallization as oxyhemoglobin the protein was precipitated as carboxyhemoglobin with a mixture of CO<sub>2</sub> and CO.

*Method 3. Electrodialysis*—A modification of the method of Stadie and Ross (11) was used. As observed by Ettisch and Groscurth (12), during electrodialysis against distilled water, the pH changes markedly and this appears to cause inhomogeneity of the hemoglobin, for when the pH is suitably controlled, crystals with fairly constant and reproducible solubility in water and

<sup>1</sup> The author is indebted to Dr. Lyon of the Lederle Laboratories for the supply of fresh horse cells.

<sup>2</sup> All pH determinations were made on a glass electrode, modified from the apparatus described by Rosebury (8). For the albumin solutions an acetate buffer 0.1 N in NaAc and 0.1 N in HAc was used as standard (pH = 4.63 (9)); for hemoglobin solutions a Clark and Lubs phosphate buffer of pH 7.0 (9). All pH values are accurate to  $\pm 0.05$  pH unit.

glycine solutions can be obtained. It was not found possible to accomplish this merely by the use of two different membranes, as recommended by Ettisch and Groscurth (12). Therefore during electrodialysis small amounts of acid or alkali were added to the outer compartments whenever necessary, to maintain the pH between the limits of 6.6 and 7.1. Variations in pH were decreased by the use of a small, narrow cell, a low current density, and by preliminary dialysis against tap water.

TABLE I  
*Carboxyhemoglobin Preparations*

Preparation	Cell Lot No.	Method of preparation
I. First dialysis preparation	1	Method 3
II-A. First HCl " "	2	Crystallized first time by CO <sub>2</sub> and O <sub>2</sub> (Method 2), recrystallized once by Method 1
II-B. " CO <sub>2</sub> " "	2	Method 2, one recrystallization
II-C. Second dialysis preparation	2	" 3
III-A. Second HCl preparation	3	" 1, two recrystallizations
III-B. " CO <sub>2</sub> " "	3	" 2, " "
III-C. Third dialysis preparation	3	" 3
III-D. HCl-dialysis preparation	3	Crystallized first by Method 1 with one recrystallization, then recrystallized once by Method 3
III-E. Dialysis-HCl " "	3	Prepared by Method 3, then recrystallized once by Method 1

The first crystallization was brought about by electrodialysis of the hemolyzed cells. For recrystallization, the hemoglobin was dissolved in N NaCl and precipitated, the first time by plain dialysis against running tap water, the second time by plain dialysis followed by electrodialysis.

The various preparations made by these methods are numbered and named as shown in Table I.

#### EXPERIMENTAL

In the determination of the activity coefficient of glycine the method of Robinson and Sinclair (13) was used. A solution of

the substance under investigation and a solution of KCl, each in two small platinum boxes, are equilibrated in an evacuated vessel at  $25.00^{\circ} \pm 0.01^{\circ}$  until the vapor pressure of the two solutions is the same and known from the concentration of KCl. The details of the procedure were exactly as described by the above authors. Before these measurements were completed Smith and Smith (14) published their results on similar measurements in which sucrose was used as standard.

The effect of glycine on the activity of egg albumin was determined by the method used by Failey (15) with the data of Sørensen (5) on the osmotic pressure of this protein in solutions of ammonium sulfate. The egg albumin-glycine solution, contained in a collodion membrane fitted onto a rubber stopper, through which passed a long capillary tube, was suspended in a glycine solution. The pH of both inner and outer solutions was adjusted to 4.73, found empirically to be the level at which there is no difference in pH in the two solutions at equilibrium. The apparatus was shaken gently in a thermostat at  $25^{\circ}$  for 48 hours. At the end of this time, which was found sufficient for equilibrium, weighed samples of inner and outer solution were removed for analysis and pH determination. The outer solution was analyzed directly for nitrogen by the micro-Kjeldahl method. The inner solution was analyzed by heat coagulation of the protein in the presence of acetate buffer, as described by Sørensen for the analysis of mixtures of egg albumin and ammonium sulfate (5), except that the protein nitrogen was determined from the difference between total and filtrate nitrogen. The albumin nitrogen factor used was 6.39 (5).

Before the solubility of hemoglobin in glycine solutions was determined, the hemoglobin was brought to approximately constant solubility by repeated rotation in the thermostat with distilled water. After each rotation nitrogen and pH determinations were made on the filtrate. The solubility of samples with added acid or alkali indicated that the preparations made by electrodialysis were at the pH of minimum solubility, but that the others were at first too acid. To hasten the attainment of constant solubility alkali was added to these until further trials showed that the pH of minimum solubility had been reached, the salts in the supernatant liquid being removed by repeated washings before equili-

brations with glycine were undertaken. The final value of pH and solubility varied considerably and the former seemed to vary with the method of preparation. All preparations made by electrodialysis, including the two recrystallized before or afterwards by Method 1, had a final pH in the range 6.90 to 6.95; Preparations II-A and II-B had pH values of 6.69 and 6.64 respectively, and Preparations III-A and III-B a pH of 6.85.

For the determination of the solubility of carboxyhemoglobin in glycine solutions enough NaOH was added to bring the pH of the final glycine solution to that of a saturated solution of hemoglobin in water. The suspensions, after saturation with CO, were rotated for 6 hours or more in the thermostat at  $25.0^{\circ} \pm 0.1^{\circ}$  and then filtered. The precipitates were used for further solubility determinations. The proportion of solid was kept roughly constant for all measurements with any one preparation.

Weighed samples of the filtrate were diluted for analysis and the concentration of glycine and hemoglobin determined in the same way as that of the glycine and albumin solutions, except that before coagulation in place of the acetate buffer a Clark and Lubs phosphate buffer of pH 6.4 (9), double strength, was added to the solution and the wash water. In the case of hemoglobin it was found that between 1 and 2 per cent of the nitrogen is not coagulable (*cf.* Sørensen and Sørensen (16)). The amount was constant for any one preparation and was always subtracted from the filtrate nitrogen before the glycine and protein concentrations were calculated. The hemoglobin was assumed to contain 16.86 per cent N (17).

### Results

*Activity Coefficient of Glycine*—The equilibrium concentrations of glycine and KCl solutions are given in Table II, where  $m_k$  is the molality of KCl and  $m_g$  the molality of glycine.

The relative molal vapor pressure lowering of KCl,  $R_k$ , for each point was read from a large scale curve of  $R_k$  as a function of  $m_k$ , constructed from the values selected by Robinson and Sinclair (13).

$$R_k = \frac{p_0 - p}{p_0 m_k}$$

## 732 Glycine Effect on Activity Coefficient

where  $p$  = vapor pressure of the solution,  $p_0$  = vapor pressure of water at 25°.

Then for glycine

$$R_g = \frac{R_h m_h}{m_g}$$

The points showed an average deviation of  $\pm 0.15$  per cent from the mean curve for  $R_g$  as a function of  $m_g$ . From this curve,

TABLE II  
*Concentrations of Isopiestic Solutions of Glycine and KCl*

$m_k$	$m_g$	$m_k$	$m_g$	$m_k$	$m_g$
0.0966	0.1821	0.6552	1.284	1.231	2.478
0.0970	0.1833	0.6632	1.297	1.244	2.519
0.1074	0.2024	0.7230	1.418	1.275	2.581
0.1087	0.2049	0.7851	1.549	1.285	2.598
0.1448	0.2737	0.7918	1.564	1.348	2.730
0.1698	0.3208	0.8302	1.641	1.357	2.756
0.1789	0.3370	0.8423	1.664	1.397	2.836
0.1842	0.3475	0.9119	1.805	1.404	2.856
0.2232	0.4236	0.9274	1.843	1.405	2.855
0.2571	0.4876	0.9639	1.917	1.408	2.870
0.2673	0.5100	0.9752	1.941	1.414	2.871
0.3003	0.5719	0.9756	1.946	1.415	2.865
0.3150	0.5997	0.9857	1.960	1.416	2.869
0.3481	0.6647	1.000	1.992	1.419	2.886
0.3628	0.6930	1.002	1.995	1.421	2.895
0.3647	0.6967	1.046	2.094	1.442	2.933
0.4115	0.7908	1.055	2.109	1.500	3.063
0.4695	0.9600	1.109	2.221	1.508	3.072
0.5523	1.100	1.118	2.246	1.540	3.143
0.5922	1.151	1.130	2.266	1.565	3.190
0.6250	1.220	1.191	2.407	1.609	3.299

drawn on a large scale, values of  $R_g$  were read at certain molalities, and from these the activity of water ( $= 1 - R_g m_g$ ) was calculated directly. To obtain the activity coefficient of the solute,  $\gamma$ , the method given by Lewis and Randall ((18) p. 273) was used. To avoid extrapolation to  $m = 0$ ,  $\log \gamma/\gamma(1M)$  was calculated from the data (cf. Table III) and  $\log \gamma(1M)$  was set equal to that (0.0638) found by Scatchard and Prentiss (3) at 0°, corrected to 25° with the data of Zittle and Schmidt (19) on

the partial molal heat content and specific heat of glycine, by the method of Lewis and Randall ((18) p. 349). When this is done, the curve for the activity coefficient of glycine, calculated from

TABLE III  
Activity Coefficient of Glycine in Water at 25°

$m_g$	$-\log \frac{\gamma}{\gamma(1M)}$	$m_g$	$-\log \frac{\gamma}{\gamma(1M)}$	$m_g$	$-\log \frac{\gamma}{\gamma(1M)}$
0.2	-0.0509	1.2	+0.0094	2.4	+0.0500
0.4	-0.0356	1.4	+0.0179	2.6	+0.0551
0.6	-0.0223	1.6	+0.0255	2.8	+0.0594
0.8	-0.0105	1.8	+0.0323	3.0	+0.0634
1.0	0	2.0	+0.0386	3.2	+0.0669
		2.2	+0.0446		

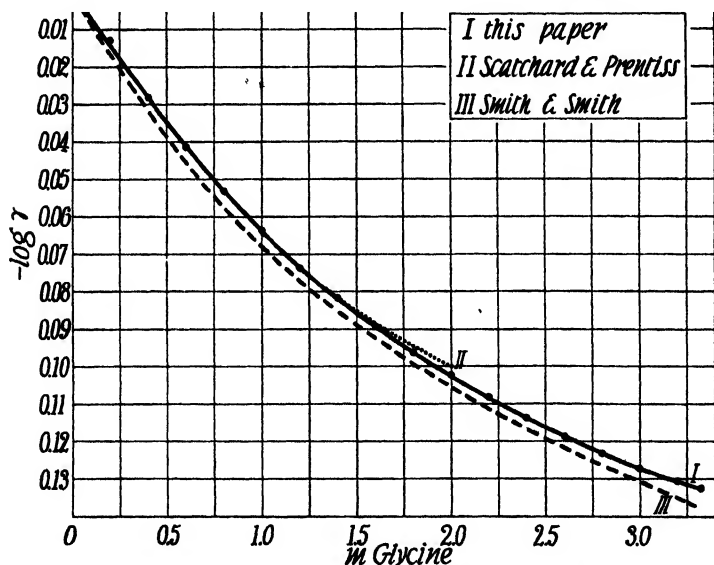


FIG. 1. Activity coefficient of glycine at 25° from vapor pressure and freezing point determinations.

the vapor pressure data, is practically identical with that from freezing point data, corrected to 25° up to 1.4 m glycine (cf. Fig. 1, Curves I and II). At higher concentrations the former falls



below, probably due partly to errors in the temperature correction for the freezing point curve. At 2 M glycine the uncertainty in the partial molal heat content is about 4 per cent, that in the partial molal heat capacity, estimated from the deviation of the points from a smooth curve, about 4 per cent, which would mean a maximum error in  $\log \gamma$  of 0.0013, or more than half the difference observed at 2.0 M. The curve obtained by Smith and Smith (14) is also shown in Fig. 1, Curve III. There is a fairly constant difference of 0.0035 between the values of  $\log \gamma$  found in this paper and those given by Smith and Smith. This is largely due to the difference in methods of obtaining the value of  $\log \gamma$  re-

TABLE IV  
*Effect of Glycine on Activity Coefficient of Egg Albumin*

$m_3 \times 10^3$	$m_2$	$m_2^*$
2.16	0.02508	0.02485
2.47	0.0502	0.0494
2.22	0.1041	0.1024
2.50	0.1120	0.1113
2.36	0.1120	0.1114
2.73	0.1270	0.1253
2.45	0.1619	0.1585
2.32	0.1684	0.1663
2.38	0.3012	0.2973
2.36	0.4885	0.4797
2.01	0.7810	0.7725
2.20	0.908	0.898
1.91	1.326	1.308

ferred to infinite dilution. These authors extrapolated their own data to infinite dilution instead of fitting them to a curve for which data were available at very low concentrations. There is also a small difference in shape, for the divergence is smaller in the center than at either end of the curves.

*Activity Coefficient of Egg Albumin in Presence of Glycine*—The molalities of glycine in the inner,  $m_2$ , and outer,  $m_2^*$ , solutions and the molality of the albumin,  $m_3$ , in the inner solution, assuming a molecular weight of 34,500 for the latter, are given in Table IV. It can be seen that the difference between the glycine molality of inner and outer solutions is only about 1 per cent of the total

glycine concentration. This is just at the limit of error of the analysis (about  $\pm 0.7$  per cent). However, since the difference is consistently in one direction in solutions of albumin concentration near  $2.3 \times 10^{-3} m$  ( $m_2^*$  always greater than  $m^2$ ), but in the opposite direction and smaller in albumin solutions of lower concentrations, and quite irregular when the pH of inner and outer solutions differed, it seems probable that the differences are not due entirely to analytical error, but that glycine does have a small salting-out effect. The results have significance only in setting a lower and an upper limit to the effect, since  $\gamma_3$ , the activity coefficient of albumin, depends largely on the difference between  $m_2^*$

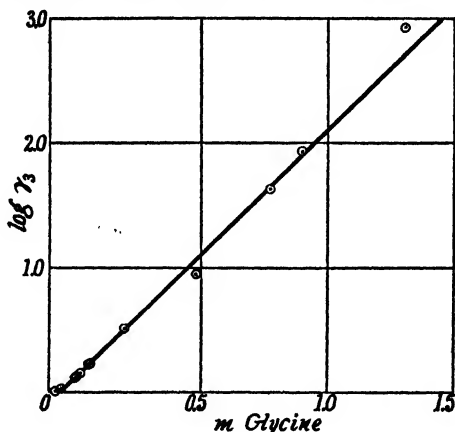


FIG. 2. Activity coefficient of egg albumin in the presence of glycine

and  $m_2$ . The slope of the curve for  $\log \gamma_3$  might be anywhere between zero and twice the calculated value and still be within the limits of experimental error.

$\log \gamma_3$  has been calculated by the method given by Failey (15).

$$\int d \log \gamma_3 = \int \frac{55.51 \bar{V}_1 + m_2 \bar{V}_2}{2.3 m_3 RT} dP + \int \frac{m_2^* - m_2}{m_3} d \log a_2^* - \int d \log m_3$$

The symbols have the same significance as in the above reference.

The contribution of the combined first and third terms is negligible; therefore,  $\log \gamma_3$  has been estimated from the second term by graphical integration. The results are shown in Fig. 2. The data fit a straight line of slope  $d \log \gamma_3 / dm_2 = 2.1$ .

*Effect of Glycine on Solubility of Carboxyhemoglobin*—The solubilities of the different hemoglobin preparations and the corresponding glycine molalities are given in Table V. Since preliminary experiments indicated that the solubility of hemoglo-

TABLE V  
*Solubility of Carboxyhemoglobin in Presence of Glycine*

$m_g$	$S$ per kilo $H_2O$	pH	$m_g$	$S$ per kilo $H_2O$	pH	$m_g$	$S$ per kilo $H_2O$	pH
Preparation I. $S_0 = 17.8$			Preparation II-C. $S_0 = 15.8$			Preparation III-C. $S_0 = 16.6$		
0.0577	19.5	6.98	0.128	21.6		0.425	34.4	6.88
0.0662	20.3	6.97	0.175	21.4	6.92	0.702	46.0	6.90
0.108	22.4	6.97	0.367	30.0	6.95	0.907	51.1	6.88
0.125	23.9	6.92	0.550	34.2	6.93	1.20	57.6	6.90
0.220	27.8	6.97	0.656	40.8	6.95	1.51	59.7	6.90
0.247	29.8	6.92	0.782	44.9	6.95	1.86	57.8	6.90
0.334	32.4	6.97	0.879	46.0	6.92	2.19	48.8	6.90
0.384	35.6	6.92	1.22	54.8		2.59	44.7	6.90
0.441	37.7	6.97	1.55	58.9	6.92	Preparation III-D. $S_0 = 13.9$		
0.498	41.2	6.92	1.63	56.0	6.94			
0.555	42.6	6.97	1.64	57.4	6.95	0.410	27.1	6.84
0.629	46.5	6.92	1.82	55.0	6.95	0.691	38.2	6.90
0.757	47.9	6.97	2.12	54.6	6.95	0.897	41.2	6.84
0.888	53.6	6.98	2.25	51.2	7.00	1.15	47.9	6.90
0.969	55.0	6.98	2.78	42.5		1.44	50.6	6.84
0.996	56.0	6.97	3.12	36.9	6.91	1.76	52.2	6.89
1.08	57.3	6.90	3.17	33.3	6.95	2.13	47.2	6.86
1.53	64.2	6.90				2.52	42.2	6.89
1.57	65.4	6.97				Preparation III-E. $S_0 = 16.4$		
1.65	63.3	6.97						
1.69	62.8	6.98				0.409	34.7	6.89
1.87	60.6	6.97				0.676	46.0	6.88
2.05	57.6	6.91				0.880	52.4	6.89
2.31	54.3	6.97				1.16	59.4	6.88
2.44	48.9	6.97				1.45	61.6	6.89
2.87	41.9	6.97				1.75	63.5	6.89
3.03	39.0	6.97				2.12	57.6	6.89
						2.48	54.7	6.89

bin was to a small extent dependent on the amount of solid present, as found by Sørensen and Sørensen (16), the proportion of solid was kept approximately constant for all points in the curve for each preparation and several determinations of the solubility

in water were always made with this same amount of solid. Therefore the ratio  $S/S_0$ , where  $S$  is the solubility in glycine solution and  $S_0$  the average water solubility for the preparation in question, is of more significance than the ratio of  $S$  to the average water solubility for all preparations. The value  $S_0$  found for each preparation is given in Table V.

When  $\log S/S_0$  is plotted against the glycine molality (cf. Fig. 3), the points for those preparations made by electrodialysis lie on the same curve, very closely up to 2 M glycine, with scatter-

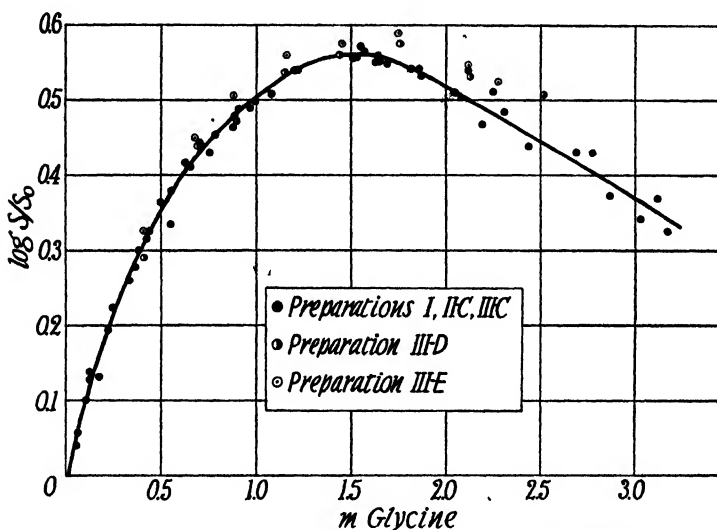


FIG. 3. Influence of glycine on the solubility of carboxyhemoglobin prepared by electrodialysis.

ing at higher concentrations. This spread is due in part to the greater analytical error in  $S$  where the glycine nitrogen is much greater than the protein nitrogen. The HCl-dialysis curve is practically identical with these up to 2 M glycine and the dialysis-HCl only slightly higher, so that the electrodialysis method of preparation apparently gives crystals reproducible as to their behavior in glycine solutions. This is true if the previous treatment is varied, but the behavior of the crystals is slightly altered if subsequently they are recrystallized by Method 1.

The preparations made by the other methods show no such

regularity (*cf.* Fig. 4). There is some indication that these results may be due to a more complete removal of impurities by the electro dialysis method. The curves for Preparations III-A and III-B, made from cells containing pyrophosphate, are much higher than those for Preparations II-A and II-B, but there is no such difference between the electro dialysis preparations from different lots of cells. During preliminary rotation to constant solubility the solubility of all the dialysis preparations changed by less than 10 per cent. The solubility of the others decreased between 30 and 80 per cent before reaching a constant value.

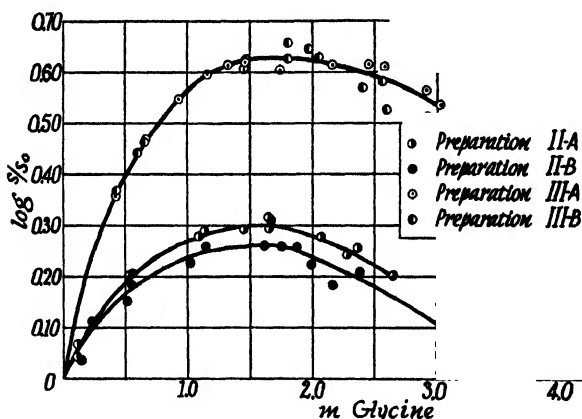


FIG. 4. Influence of glycine on the solubility of carboxyhemoglobin prepared by Methods 2 and 3.

Possibly some of the impurities in the preparations which were not electro dialyzed are incompletely removed salts or decomposition products produced by treatment with alkali and acid.

Because of the variability in the others, only the curve for the electro dialyzed hemoglobin is considered in detail. Its limiting slope

$$\frac{d \log S/S_0}{dm}$$

at low glycine concentrations is 1.05; at high, approximately -0.16.

## DISCUSSION

The above results indicate that the effect of glycine upon itself or other zwitter ions is analogous to that of salts on these compounds. For glycine alone, the salting-in effect is marked at low concentrations, a salting-out effect only indicated by decreased slope at high ones. Similarly, a decrease in activity coefficient occurs on addition of salts, but little or no salting-out effect can be detected even in concentrated salt solutions (20). On the other hand, with the very soluble protein egg albumin, only a doubtful salting-out effect could be demonstrated in the presence of glycine; with  $(\text{NH}_4)_2\text{SO}_4$  a large salting-out at high salt concentrations and only a small salting-in at low ones (15) have been observed. With carboxyhemoglobin increase in solubility in dilute solution of added substance and decrease in concentrated solution are marked in solutions of both glycine and salt (7). Thus it seems that the factors determining whether the activity coefficient of a particular zwitter ion will be more readily decreased or increased are similar for glycine and salts.

It has been repeatedly shown (7, 15, 21) that the activity coefficient of proteins in the presence of concentrated salts increases linearly with the salt concentration. The same relation seems to hold for concentrated glycine and proteins within the limits of error in the data in this paper on the activity coefficients of hemoglobin and albumin in the region where the salting-out effect predominates.

For dilute solutions, Kirkwood (22) has developed a theory to account for the change in the activity coefficient of zwitter ions due to change in dielectric constant of the solvent. The theory predicts that when the dielectric constant,  $D$ , is large,  $\log \gamma$  should be directly proportional to  $1/D$ . In an attempt to see how far the effect of glycine on itself and hemoglobin might be interpreted as that of a solvent of increased dielectric constant, the results on glycine and hemoglobin were plotted in this way (see below) and were found to fit this linear relation surprisingly well, in spite of the fact that the theory should apply only to dilute solutions in which the mutual electrostatic interaction of the zwitter ions can be neglected. This may indicate that a large part of their interaction is governed by the same forces as is their

interaction with any other non-electrolyte which alters the dielectric constant of the medium.

The dielectric constants of the glycine solutions and of the hemoglobin-glycine solutions below 1.8 M glycine were calculated from Wyman's values for the dielectric constant of water,  $D_0$ , and the dielectric increment,  $\delta$ , of glycine at 25° (23) and from the approximate value of 30,000 per mole (1) for the dielectric increment of hemoglobin. In calculating  $D (= D_0 + \delta_0)$ , the concentration in moles per liter must be used. This was obtained from the observed molalities and the apparent partial molal volume of the solutes (24, 25), assuming these to be independent of each other

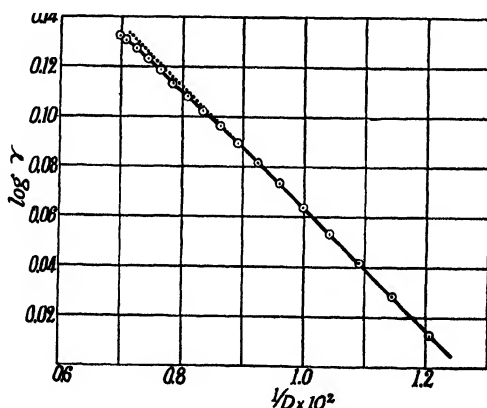


FIG. 5. Activity coefficient of glycine as a function of the dielectric constant of the solution.

in the glycine-hemoglobin solutions. The partial specific volume of hemoglobin was taken as 0.75. The results of these calculations are shown in Figs. 5 and 6.

It can be seen that the logarithm of the activity coefficient of glycine, plotted against  $1/D$ , lies on a straight line up to about 1.7 M and then falls off slightly. For hemoglobin the curve of  $\log S/S_0$  plotted against  $1/D$  approaches a straight line only in the most dilute glycine solutions (below 0.2 M), but the curvature is much less than when  $\log S/S_0$  is plotted against the molality. Qualitatively therefore the effect of glycine in decreasing the activity coefficient of these two zwitter ions may be considered as

that of a solvent of increased dielectric constant. But quantitatively Kirkwood's theory in the limiting form does not apply even to the data on glycine alone. No attempt has been made to apply it to the hemoglobin solubility data, since the theory was derived for a spherical dipole and hemoglobin is neither spherical (26) nor a dipole. However, it may be observed that

$$\frac{d \log \gamma}{d(1/D)}$$

is larger for hemoglobin (=190) than for glycine (=23.4). Since the radius of hemoglobin is larger, this implies a greater total mo-

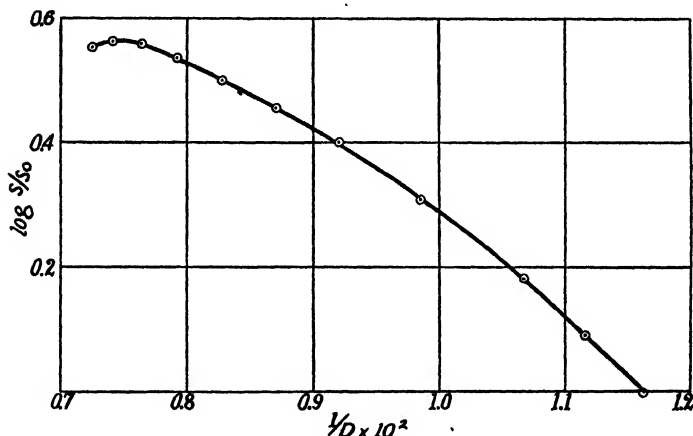


FIG. 6. Solubility of carboxyhemoglobin as a function of the dielectric constant of the solution.

ment (see below), as is indicated also by its large dielectric increment (1).

The first term of Kirkwood's equation for the change in activity coefficient with change in dielectric constant is

$$\frac{d \log \gamma}{d(1/D)} = \frac{2.374 \times 10^{15} \mu^2}{T} \frac{1}{b^3 (1 + \frac{1}{2}D)^2}$$

where  $T$  = absolute temperature,  $\mu$  = dipole moment of zwitter ion,  $b$  = radius of zwitter ion,  $\gamma$  = activity coefficient of zwitter ion, and  $D$  = dielectric constant of solution.

Since  $1 + (1/2D)$  is close to unity, the slope should be propor-



tional to  $\mu^2/b^3$ , but should increase slightly with increase in dielectric constant. Actually it decreases. From Cohn's data on the solubility of glycine in LiCl (27), Kirkwood estimated the value of  $\mu$  as  $15 \times 10^{-18}$  electrostatic units. Substituting this in the above equation gives  $b = 4.2\text{\AA}$ ., which is of the right order of magnitude, though larger than the value 2.6 to 2.8 $\text{\AA}$ . estimated from the solubility of glycine in alcohol solutions and from its molal volume (22).

That the above theory does not completely describe the data is to be expected. In the first place, the theory was derived only for dilute solutions. In the second place, it has been shown (28) that in various solvents, formamide, acetone, and the alcohols, the logarithm of the solubility of various amino acids is not even qualitatively proportional to the inverse of the dielectric constant, but that specific properties of the solvent enter in. Finally the solubility of amino acids and peptides in alcohol-water solutions (28, 29) indicates that specific properties of these compounds related to groups other than the zwitter ion group are involved in their behavior.

These specific effects and the consequent departure from theoretical behavior are greater the larger the molecule. Thus  $\log S/S_0$  in the case of hemoglobin is a linear function of  $1/D$  only in the most dilute glycine solutions.

#### SUMMARY

The activity coefficient of glycine at 25° has been determined from the relative vapor pressure lowering of its solutions and has been found to be a linear function of the inverse of the dielectric constant of the solution up to a concentration of 1.7 m.

The effect of glycine on the activity coefficient of egg albumin has been determined from distribution measurements. A salting-out effect, almost within the limits of error of the data, was noted.

A method is described for the preparation of carboxyhemoglobin crystals of reproducible solubility. Its solubility in the presence of glycine was measured and found to reach a maximum around 1.6 m.

The author wishes to express her gratitude to Professor Crawford F. Failey for suggesting this investigation and for his advice and interest throughout.

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## PROTEINS OF THE BLACK BEAN OF THE MAYAS, *PHASEOLUS VULGARIS*

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There came recently to our hands a sample of black beans which Dr. S. G. Morley of the Smithsonian Institution had collected in Yucatan, where they are used as the principal supplement to maize in the diet of the people. The Mayas, he reported, subsist almost entirely on maize and beans, and are a sturdy, healthy race. A bush bean probably identical with the beans Dr. Morley collected in Yucatan is grown in South America and used extensively for food. It is there referred to as the black bean, and its history dates back to olden days.<sup>1</sup>

Dr. V. R. Boswell of the Bureau of Plant Industry kindly identified the sample referred to as *Phaseolus vulgaris*, and advised that they are known as the "black turtle soup" bean. They are apparently identical in type with a large number of specimens of black turtle soup bean introduced from Guatemala about 15 years ago and are similar in properties to the kidney bean. Although there is not much trade in them in the United States, they are on the market to some extent but are not popular because they are a black bean.

A quantity of the beans sufficient for a study of their proteins was received from the Division of Plant Exploration and Introduction through the kind offices of Dr. F. D. Richey of the Bureau of Plant Industry. These beans were shipped from Costa Rica and were used for the work described in this paper.

In view of the reported extensive use of these beans together

<sup>1</sup> Personal communication from Dr. W. E. Whitehouse, Bureau of Plant Industry, United States Department of Agriculture, Washington.

with maize in the diet of the Mayas, it was considered of interest to study the possible supplementary relationship that may exist between their proteins and those of maize with respect to amino acid composition, and to compare their properties and composition with those of other varieties of beans which have been studied.

Most legume seeds contain two types of globulins which have been designated  $\alpha$ -globulins and  $\beta$ -globulins (1). These globulins are differentiated primarily by their solubilities, coagulation temperatures, and amino acid composition. Strikingly characteristic are their differences in cystine content. The cystine content of the  $\alpha$ -globulins ranges from 7 to 8 times that of the  $\beta$ -globulins. Usually the  $\beta$ -globulins constitute by far the greater part of the total globulin of the seeds.

It is seldom that a definite concentration of ammonium sulfate can be used which will effect a sharp separation of the two globulins. Generally a middle fraction from the apparent upper limit of the first main precipitate to the apparent lower limit of the second is removed before the latter is precipitated. This middle fraction is generally found to be a mixture of the two globulins.

Preliminary tests with NaCl solutions showed that the maximum amount of nitrogen was removed by a 2 per cent solution of this salt. 5 cc. of the solvent per gm. of meal were used, and the extraction was carried on for 2 hours with stirring. Repeated extractions removed 82 per cent of the total nitrogen of the meal. Most of the  $\alpha$ -globulin was precipitated from the extract at 35 per cent of saturation with ammonium sulfate. The fraction that further separated up to 55 per cent of saturation consisted of a mixture of the  $\alpha$ - and  $\beta$ -globulins. Increasing concentration of ammonium sulfate up to 80 per cent of saturation gave a heavy precipitate of the  $\beta$ -globulin. Additional ammonium sulfate caused no further precipitation.

Conclusive evidence of the presence of albumin or proteose in the extract was not found in the preliminary tests or in the subsequent part of the study.

#### *Preparation of Proteins*

The beans were ground to a fine meal in a power-driven mill. Care was taken to avoid heating. The meal contained 3.81 per cent nitrogen.

2 kilos of freshly ground meal were extracted with 10 liters of 2 per cent NaCl solution for 2 hours at room temperature with frequent stirring. The mixture was centrifuged, and the residue was washed once with 2 per cent NaCl solution. The clear, dark purplish extract had a reaction of pH 7.0. The combined extract and washing measured 11 liters.

*$\alpha$ -Globulin*—The  $\alpha$ -globulin was precipitated from the clear extract of the meal by addition of 26.7 gm. of ammonium sulfate per 100 cc. of extract (35 per cent of saturation). The globulin was separated by centrifugation and was washed five times with 2 per cent NaCl solution containing the same concentration of ammonium sulfate as that from which it was precipitated. The combined centrifugates were reserved for the preparation of the  $\beta$ -globulin. The washed  $\alpha$ -globulin was dispersed in 2 liters of 2 per cent NaCl solution and the filtered solution was dialyzed for 18 days against running cold water. Toluene was used as preservative. The globulin which precipitated was washed and dried with alcohol and ether<sup>2</sup> in the usual way (Preparation I). The preparation weighed 4 gm. and consisted of a dark, drab powder.

The highly opalescent dialysate from the  $\alpha$ -globulin had a reaction of pH 7.1. It became turbid but yielded no coagulum when slowly heated at 84–90°. Addition of a small amount of acetic acid after it reached this temperature, enough to bring the solution to pH 6.8, caused the separation of a heavy coagulum. Strangely, however, when the solution was adjusted to pH 6.8 before heating, no coagulum formed on subsequent heating. The coagulum was washed and dried in the usual manner (Preparation II). It consisted of a brownish drab powder,<sup>3</sup> and weighed 6 gm. Analysis of this preparation showed it to be practically identical in composition with Preparation I, indicating that it was a slightly changed form of the  $\alpha$ -globulin, a change probably brought about during the dialysis by enzymatic action.

<sup>2</sup> Evaporation of the ether washings yielded a small quantity of a heavy, oily substance. This was examined by Dr. R. S. McKinney of the Oil, Fat and Wax Laboratory of this Bureau. It was found to have a saponification value of 73.3. It proved not to be a fat, and gave negative tests for carbohydrate and saturated hydrocarbon. It was believed to be of a lipid nature.

<sup>3</sup> The color of the  $\alpha$ - and  $\beta$ -globulin preparations was due to the absorption of a trace of pigment extracted from the black hulls of the beans. It had no significant effect upon their composition.

The combined weight of Preparations I and II amounted to 10 gm.

*$\beta$ -Globulin*—The intermediate fraction of mixed globulins was removed from the filtrate remaining after separation of the  $\alpha$ -globulin by further addition of 15.26 gm. of ammonium sulfate per 100 cc. of the filtrate (enough to bring it up to 55 per cent of saturation). The precipitate was removed by filtration on a pad of filter paper pulp which had been moistened with a 55 per cent saturated solution of ammonium sulfate.

There were then added to the filtrate from the middle fraction 19.08 gm. of ammonium sulfate per 100 cc. to bring it up to 80 per cent saturation. The precipitated  $\beta$ -globulin was filtered off on folded filter papers. The globulin was then dispersed in 2 liters of distilled water. The amount of ammonium sulfate adhering to the moist precipitate was sufficient to effect solution without the use of additional salt. The solution was filtered and was dialyzed for 19 days. The precipitated protein was washed, and dried in the usual manner with alcohol and ether. The product consisted of a light, cream-colored powder, which weighed 62 gm. (Preparation III).

This amount of material did not account for nearly all of the  $\beta$ -globulin which had been precipitated by ammonium sulfate. The dialysate was, therefore, carefully examined for other protein material. When heated to 65–68° a coagulum separated. Analysis of the washed and dried coagulum (Preparation IV) gave results closely agreeing with those obtained for Preparation III. This preparation doubtless represents a slightly changed form of the  $\beta$ -globulin produced as the result of enzymatic action during the rather long period of dialysis to which it had been subjected. This product weighed 18 gm., which together with Preparation III amounted to 80 gm. of the  $\beta$ -globulin.

#### *Properties of Globulins*

*$\alpha$ -Globulin*—The  $\alpha$ -globulin of the black bean is readily dispersed in 2 per cent NaCl solution, from which it is precipitable by addition of sufficient ammonium sulfate to make the solution 35 per cent saturated with this salt. It cannot be reprecipitated from the salt solution at room temperature by dilution with 20 volumes

of water. It precipitates from the diluted solution, however, after standing for several hours at 10°. It coagulates at 75° in a 2 per cent NaCl solution acidified with acetic acid to pH 6.8. A neutral salt solution of the globulin, however, becomes only densely turbid at 90° but does not flocculate.

Like the  $\alpha$ -globulins of other beans of the *Phaseolus* genus, the  $\alpha$ -globulin of the black bean is characterized by a lower nitrogen content, and a much higher cystine and tryptophane content, than that of the  $\beta$ -globulin.

The dried  $\alpha$ -globulin was obtained as a brownish drab powder.

*$\beta$ -Globulin*—The  $\beta$ -globulin readily disperses in 2 per cent NaCl solution, and can be reprecipitated by addition of ammonium sulfate to 80 per cent of saturation. When the NaCl solution of the globulin is diluted with 20 volumes of water at room temperature, the mixture becomes milky. The protein precipitates after standing for several hours in a refrigerator. Without acidification it only partially coagulates in 2 per cent NaCl solution at 90°, but it is completely precipitated at this temperature after the solution has been adjusted to a reaction of pH 6.8 by addition of acetic acid.

The dried preparation as obtained consisted of a light cream-colored powder.

### *Analyses of Protein Preparations*

The nitrogen, sulfur, ash, and moisture contents of the  $\alpha$ - and  $\beta$ -globulin preparations are given in Table I. The distribution of nitrogen in the proteins as determined by the Van Slyke method (2) is shown in Table II.

In Table III are given the percentages of some of the amino acids found in the proteins. Cystine was determined by the method of Sullivan (3), tryptophane by the method of May and Rose (4), and tyrosine according to the method of Folin and Ciocalteu (5). The figures for arginine, histidine, and lysine were calculated from the results of the Van Slyke analyses given in Table II.

The percentages given in Tables I to IV have been calculated on the basis of ash- and moisture-free protein.

For comparison of the data found for the proteins of the black bean with those similarly obtained for other beans of the same



genus, there are given in Table IV figures previously determined on the proteins of the navy bean (1), mung bean (6), adzuki bean (7), and the Lima bean (8).

TABLE I  
*Elementary Composition of  $\alpha$ - and  $\beta$ -Globulins of Black Bean*

	$\alpha$ -Globulin		$\beta$ -Globulin	
	Preparation I	Preparation II	Preparation III	Preparation IV
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Nitrogen*.....	15.60	15.71	16.57	16.62
Sulfur*.....	1.03	1.03	0.27	0.28
Ash.....	0.61	0.35	1.24	0.75
Moisture.....	10.21	8.85	8.27	9.53

\* Percentages calculated on the basis of ash- and moisture-free material.

TABLE II  
*Distribution of Nitrogen in  $\alpha$ - and  $\beta$ -Globulins*

	$\alpha$ -Globulin*	$\beta$ -Globulin	
	Preparation II	Preparation III	Preparation IV
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Amide N.....	9.86	10.98	10.72
Humin " adsorbed by lime.....	3.03	2.78	2.67
" " in ether-amyl alcohol-extract.....	0.42	0.53	0.45
Arginine N.....	10.96	12.12	12.07
Cystine N.....	0.91	0.89	0.85
Histidine N.....	4.41	5.47	5.39
Lysine N.....	7.81	7.76	7.78
Amino " of filtrate.....	60.97	56.07	57.06
Non-amino N of filtrate.....	2.08	3.04	2.62
Total N regained.....	100.45	99.64	99.61

\* Because of insufficient material the distribution of nitrogen was not determined on Preparation I.

In general, there is quite a close agreement in composition between the corresponding proteins of the different beans. This is particularly true with respect to their nitrogen and sulfur content. Tyrosine and lysine are present in relatively large amounts

in both globulins of all the beans. The  $\alpha$ - and  $\beta$ -globulins of the black bean, however, contain appreciably less lysine than the corresponding globulins of the other beans, with the exception of

TABLE III  
*Amino Acids in  $\alpha$ - and  $\beta$ -Globulin Preparations*

Amino acid	$\alpha$ -Globulin		$\beta$ -Globulin		Zein
	Preparation I	Preparation II	Preparation III	Preparation IV	
	per cent	per cent	per cent	per cent	per cent
Arginine.....		5.34	6.24	6.23	1.8
Histidine.....		2.56	3.34	3.30	0.8
Lysine.....		6.39	6.71	6.75	0.0
Cystine.....	0.77	0.77	0.11	0.12	0.8
Tryptophane.....	2.94	3.04	0.98	0.99	0.17
Tyrosine.....	5.05	5.10	4.25	4.27	5.9

TABLE IV  
*Comparison of Globulins of Black Bean with Those of Other Beans of Phaseolus Group*

The values are expressed as percentages of the proteins.

	$\alpha$ -Globulins					$\beta$ -Globulins				
	Black turtle soup bean	Navy bean	Mung bean	Ad-suki bean	Lima bean	Black turtle soup bean	Navy bean	Mung bean	Ad-suki bean	Lima bean
Nitrogen.....	15.71	15.65	15.67	15.60	15.55	16.57	16.30	16.83	16.57	14.81
Sulfur.....	1.03	1.38	1.50	1.21	1.27	0.27	0.33	0.41	0.40	0.35
Cystine*.....	0.77	0.79	1.37	0.51	1.15	0.11	0.12	0.06	0.13	0.12
Arginine.....	5.34	6.87	5.13	5.45	5.67	6.24	6.36	7.56	7.00	5.07
Histidine.....	2.56	0.85	3.30	2.25	3.71	3.34	2.36	2.02	2.51	2.62
Lysine.....	6.39	10.69	6.08	8.30	7.84	6.71	9.42	9.29	8.41	8.53
Tryptophane....	3.04	2.79	2.03	1.72	1.92	0.98	0.94	1.18	0.96	2.16
Tyrosine.....	5.10	4.95	3.70	4.28	4.97	4.25	4.16	4.84	3.91	4.27

\* All the cystine determinations were made colorimetrically by the method of Sullivan (3).

that for the  $\alpha$ -globulin of the mung bean. The  $\beta$ -globulins of all the beans are characteristically low in cystine. The histidine content of the  $\alpha$ -globulin of the black bean is 3 times that of the corresponding globulin of the navy bean, but about one-third

less than that of the Lima and mung beans. The  $\beta$ -globulin of the black bean, however, contains about one-third more histidine than the  $\beta$ -globulins of the other beans. Since the  $\beta$ -globulins constitute by far the greater proportion of the total protein of the seeds, it would appear that the black bean is a considerably better source of histidine than the other beans which have been considered. The tryptophane value of the total globulins of the black bean closely approximates that of the other beans.

The biological value of a protein depends not only upon its absolute content of the dietary essential amino acids, but also on the extent to which they can be utilized by the body for nutritional requirements. Digestion studies, both *in vitro* and *in vivo*, have showed that the proteins of practically all the beans of the *Phaseolus* genus have a low coefficient of digestibility when in the raw state. Rats will not grow satisfactorily when raw navy bean meal, for example, or its isolated proteins constitute the sole source of protein in an otherwise adequate diet, even after supplementation with cystine, unless the proteins have been heated sufficiently to coagulate them. Although no data are available on the digestibility of the black bean proteins, the fact that its proteins agree so closely in chemical composition with those of other beans botanically related makes it appear very probable that they also share with them the property of indigestibility when in the raw state.

The large extent to which the diet of the Mayas consists of maize and the black bean lends interest to a comparison of the amino acid content of the proteins of these two seeds from a consideration of the possible supplementary value of their proteins. To what extent do the proteins from the one source supply a liberal amount of the dietary essential amino acids which are lacking or deficient in the other?

Of the amino acids determined in the proteins of the black bean, lysine, histidine, and tryptophane are generally recognized as dietary essential amino acids. Until recently, cystine has also been regarded as dietary essential. Jackson and Block (9) and Rose *et al.* (10) have shown, however, that cystine can be replaced in the diet by methionine. Little, if any, information is available on the methionine content of the proteins of beans. Whatever

may be the relationship between these two amino acids in the nutritional properties of the proteins of legume seeds, it has been amply demonstrated by different workers that cystine is a limiting factor.

Rose and coworkers have recently added several other amino acids to the list of those which are dietary essential. These include leucine, isoleucine, phenylalanine, threonine, and valine. In considering the supplementary relationship between different proteins, the percentages of these amino acids should also be compared, but at present this is not practical because sufficiently accurate data on the amounts present in proteins are not available.

In comparing the proteins of the black bean with those of maize from the standpoint of supplemental values, emphasis should be laid on the relative amounts of lysine, cystine, and tryptophane present, for these amino acids are known to be limiting factors in the proteins of one or the other of these seeds. In Table III are given for comparison figures showing the percentages of amino acids in zein, the chief protein of maize.

The lysine content of the proteins of the black bean like that of most legume seeds exceeds that of most food proteins. Zein is devoid of this amino acid. The proteins of the black bean have practically the same low content of cystine as the corresponding proteins of the navy bean, which are inadequate for supporting growth unless supplemented with cystine. The cystine content of zein is a little higher than that of the  $\alpha$ -globulin of the black bean and about 7 times that of the  $\beta$ -globulin, which is the predominating protein of the bean. There is no evidence, as far as we are aware, that cystine is a limiting factor in the total proteins of maize.

Zein is conspicuously lacking in tryptophane. There is a fair amount of tryptophane in the  $\beta$ -globulin of the black bean, and more in the  $\alpha$ -globulin than in most proteins. The amount of histidine in zein is also much lower than in the black bean proteins.

It is realized that the above comparisons are made between two globulins of the black bean which comprise the greater part of its total protein content, and zein which represents only one of the maize proteins. Other proteins occur in maize in smaller pro-

portions, but not much is known concerning their amino acid composition. However, it is believed that the total proteins of maize have the same amino acid deficiencies as zein, although to a lesser degree. Hogan (11) has shown by feeding experiments that the total proteins of maize do not supply enough lysine or tryptophane to meet the growth requirements of the rat and that tryptophane is the first limiting factor.

On the basis of the experimental data presented it is not believed that a significant superiority in nutritive value can justly be claimed for the proteins of the black bean over those of most of the other beans which have been considered. In general, there is a striking similarity between the different varieties of the *Phaseolus* beans with respect to the chemical and physical properties of their proteins. It would seem that a supplemental relationship exists between the proteins of maize and those of all these beans. The relatively higher content of histidine in the black bean proteins may, however, entitle them to the claim of some preference.

#### SUMMARY

The chief proteins of the black turtle soup bean, a variety of *Phaseolus vulgaris*, consist of an  $\alpha$ - and a  $\beta$ -globulin. These globulins have been isolated and a study made of their nitrogen distribution and amino acid content. They are strikingly similar in their chemical and physical properties to the corresponding proteins of other beans of the *Phaseolus* genus.

It is reported that the Mayas subsist almost entirely on maize and this bean, and are a sturdy, healthy race. A comparison of the amino acid composition of the black bean proteins with those of maize indicates a supplemental relationship. The relatively large amount of lysine, tryptophane, histidine, and cystine in the bean proteins is in marked contrast to that of maize.

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# THE DETERMINATION OF SULFANILAMIDE IN TUNGSTIC ACID BLOOD FILTRATES BY MEANS OF SODIUM $\beta$ -NAPHTHOQUINONE-4-SULFONATE

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Blood sulfanilamide determinations are frequently requested in the hospital laboratory since the introduction of this chemotherapeutic agent in the treatment of certain infectious diseases (1-3). A very good method for the analysis of this drug in blood and other body fluids has been developed (4) and utilized experimentally (5) by Marshall and his associates. While their diazotization procedure is extremely sensitive and accurate, it necessitates the preparation of a special toluenesulfonic acid blood filtrate. It would seem that an alternative method for the determination of sulfanilamide which could be carried out on the routine Folin-Wu tungstic acid blood filtrates (6) would be very helpful.

The observation that deeply colored solutions or precipitates are found in the reaction between amino compounds and sodium  $\beta$ -naphthoquinone-4-sulfonate (7-10) has been applied to the development of a very simple quantitative method for the determination of sulfanilamide in tungstic acid blood filtrates. Under the correct experimental conditions measured quantities of the drug added to blood were fully and accurately recovered. Likewise, the values given by this method for a series of bloods taken from patients undergoing sulfanilamide therapy were found to check very well with the values given by Marshall's diazotization method (4).

## EXPERIMENTAL

While there are many constituents normally present in protein-free blood filtrates which react with the  $\beta$ -naphthoquinonesulfonic



acid when in slightly alkaline solution, thus furnishing the bases for the Folin colorimetric amino acid method (10), none of them seems to react with this reagent when in acid solution. The pH of Folin-Wu filtrates, prepared according to the Haden technique (11), generally varies from 4 to 5. This degree of acidity prevents any reaction with the chromophoric reagent. This fact was readily demonstrated by adding 1 cc. portions of freshly prepared 0.05 per cent sodium  $\beta$ -naphthoquinone-4-sulfonate (Eastman, No. 1372) to 10 cc. of normal blood filtrate and to 10 cc. of distilled water. The tubes were placed in the dark at once and at various intervals, ranging from 0 to 90 minutes, matched against each other in the colorimeter. With the distilled water specimen set at 20 mm. the filtrate also read close to 20 mm. Hence normal filtrates show no evidence of color intensification under these experimental conditions. The tubes must always be placed in the dark, because light, even on a dark day, rapidly decolorizes the dilute quinone reagent in the standard or in the distilled water, whereas the blood filtrates seem to exert a definite protective action against decolorization.

The reaction between the quinone reagent and the sulfanilamide in concentrations ranging from 0.01 to 0.2 mg. per 10 cc. of solution (corresponding to 1 to 20 mg. per cent bloods) proceeds fairly rapidly, the final color ranging from a straw-yellow to an orange-red. If artificial standards, prepared from slightly acidified solutions of alcoholic methyl orange, are set at 20.0 mm. in the colorimeter, it can readily be ascertained that the color in the sulfanilamide solutions attains maximal intensity within 45 to 60 minutes. After 90 minutes the readings may show some irregularity. The reaction product is rather insoluble and in the more concentrated tubes tends to come out of solution. However, the addition of 1 drop of 0.1 N HCl to each standard and filtrate at the beginning of the test lowers the pH of each solution to about 3 and will delay flocculation for several hours.

The data in Table I indicate that the color given in the reaction between the naphthoquinone sulfonate and sulfanilamide lacks good proportionality. Similar values were given by a series of normal blood filtrates containing equivalent amounts of sulfanilamide. Hence one must either determine proportionality curves or else use several standards when analyzing unknown blood

filtrates. We have found it more satisfactory to set up at least five standards corresponding to 2, 4, 7, 10, and 15 mg. per cent bloods and to read each filtrate against the standard which it most closely matches. If necessary, any two of these standards may be mixed in order to secure the corresponding intervening standard.

*Recovery of Sulfanilamide Added to Blood*—After the routine blood chemistry determinations in the hospital laboratory were completed, the residual oxalated bloods were pooled and carefully divided into 19 cc. portions. To each flask was added exactly 1 cc. of a stock solution of a strength to yield a series of bloods ranging from 2 to 20 mg. per cent sulfanilamide. Each blood was well

TABLE I  
*Proportionality Curves Given by Sulfanilamide with  
 $\beta$ -Naphthoquinonesulfonic Acid Reagent*

Added			Recovered			Added			Recovered		
mg. per 10 cc.	mg. per 10 cc.	per cent	mg. per 10 cc.	mg. per 10 cc.	per cent	mg. per 10 cc.	mg. per 10 cc.	per cent	mg. per 10 cc.	mg. per 10 cc.	per cent
0.20	0.1709	85.45	0.10	0.0840	84.04	0.04	0.0323	80.75			
0.15	0.1399	93.27	0.08	0.0714	89.25	0.03	0.0250	83.33			
0.10*	0.1000	100.00	0.06	0.0581	96.83	0.02*	0.0200	100.00			
0.09	0.0935	103.89	0.05*	0.0500	100.00	0.015	0.0163	108.67			
0.08	0.0833	104.13	0.04	0.0444	111.00	0.01	0.0148	148.00			
0.05	0.0667	133.40	0.03	0.0384	128.00						

\* Standard set at 20.0 mm. in the colorimeter.

mixed and, after 5 to 10 minutes, a 5 cc. portion was used to prepare the Folin-Wu (6) filtrate and another 5 cc. portion used to prepare the Marshall toluenesulfonic acid filtrate. For the  $\beta$ -naphthoquinonesulfonic acid method a series of standards containing 0.02 to 0.2 mg. of sulfanilamide (Winthrop Chemical Company, Inc.) per 10 cc., corresponding to the 2 to 20 mg. per cent bloods, was set up in test-tubes and, in addition, 10 cc. portions of each of the Folin-Wu filtrates. To each standard and filtrate alike were added just 1 drop of 0.1 N HCl and 1 cc. of freshly prepared 0.05 per cent sodium  $\beta$ -naphthoquinone-4-sulfonate. The contents were mixed and all tubes immediately placed in the dark for 45 to 60 minutes, whereupon each filtrate

was read in the colorimeter against the standard which it most closely matched.

TABLE II

*Recovery of Sulfanilamide Added to Human Blood by  $\beta$ -Naphthoquinone-sulfonic Acid and Marshall's Diazotization Methods*

Sulfanilamide added	Sulfanilamide found by $\beta$ -naphthoquinone-sulfonic acid method	Error	Sulfanilamide found by Marshall's method	Error
mg. per 100 cc.	mg. per 100 cc.	per cent	mg. per 100 cc.	per cent
20.0	19.51	-2.45	19.80	-1.00
15.0	14.78	-1.47	15.39	+2.60
10.0	10.05	+0.50	9.76	-2.40
7.0	7.33	+4.71	6.86	-2.00
5.0	5.00	0.00	4.83	-3.33
3.5	3.30	-4.29	3.56	+1.71
2.0	1.98	-1.0	2.00	0.00

TABLE III

*Comparative Analysis of Blood of Patients Undergoing Sulfanilamide Therapy*

The values are given as mg. of sulfanilamide per 100 cc. of blood.

Case No.	$\beta$ -Naphthoquinone-sulfonic acid method	Marshall's method	Difference	Clinical diagnosis
1	19.1	18.7	+0.4	Meningitis, <i>Streptococcus viridans</i>
2	14.8	14.2	+0.6	Bronchopneumonia
3	14.5	15.1	-0.6	Puerperal sepsis
4	12.4	12.9	-0.5	Meningitis, <i>Streptococcus viridans</i>
5	8.4	8.1	+0.3	Pelvic peritonitis
6	6.7	6.5	+0.2	Ulcerative pharyngitis and tonsillitis, Vincent's angina
7	6.7	7.0	-0.3	Puerperal sepsis (postabortive)
8	5.0	5.3	-0.3	Encrusted cystitis
9	5.0	5.3	-0.3	" "
10	4.9	5.0	-0.1	Subacute bacterial endocarditis
11	1.6	1.5	+0.1	Bronchopneumonia, emphysema, chronic bronchitis

In a similar manner a series of sulfanilamide standards containing toluenesulfonic acid was set up, including 10 cc. portions

of each of the Marshall blood filtrates, and their drug content determined according to Marshall's directions (4). We likewise used Kahlbaum's sodium nitrite and saponin and Eastman's No. 984 toluenesulfonic acid and No. 1060 dimethyl- $\alpha$ -naphthylamine. The data are given in Table II and, as can readily be ascertained, excellent sulfanilamide recovery was secured with both methods.

Following these experiments, we obtained specimens of blood from patients undergoing sulfanilamide therapy and analyzed them by both methods. These data are given in Table III. The values given by the  $\beta$ -naphthoquinonesulfonic acid method were found to check very well with the values secured with the Marshall diazotization procedure. Marshall's procedure yields solutions of relatively greater color intensity, which follow Beer's law much more closely than do those given by the  $\beta$ -naphthoquinonesulfonic acid method. However, the fact that the latter method may be applied to routine tungstic acid blood filtrates with considerable accuracy should prove helpful in the hospital laboratory.

The possibility of some foreign compound or drug which might react with the quinone reagent in acid solution getting into the blood stream and thus producing an erroneously high sulfanilamide value seems remote. A compound of that nature would probably react with the reagent in alkaline solution also. Although we have made several thousand amino acid nitrogen determinations by the Folin colorimetric method on individual bloods, we have never seen a high value which could not be readily explained by the clinical condition of the patient (12, 13).

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#### SUMMARY

1. A simple method with sodium  $\beta$ -naphthoquinone-4-sulfonate has been developed by which the sulfanilamide content of Folin-Wu tungstic acid blood filtrates may be accurately determined.

2. The values obtained by this method on bloods secured from patients undergoing sulfanilamide therapy were found to check closely with the values obtained by Marshall's diazotization method.

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## PHYTOHORMONES: STRUCTURE AND PHYSIOLOGICAL ACTIVITY. I

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In plants, growth in length by cell elongation is conditioned by certain substances elaborated in the plant and effective in minute amounts, thus having the nature of hormones. A number of simple tests have been worked out which allow of a qualitative and quantitative determination of the growth-promoting properties of various substances. With the aid of these biological tests the native growth substances or auxins have been isolated and a variety of other substances have been shown to possess growth activity. At first sight the large number of substances which are active do not appear to fall within any general classification. Upon further investigation, however, it becomes clear that there are certain common structural characteristics, and an analysis of these provides a first step in attacking the fundamental problem of the relations between chemical structure and physiological activity. This relation has in recent years been attacked in a number of other fields, usually involving physiological processes in animals. On the other hand, it would appear that plants offer an exceptionally favorable field for study in that their structures—and possibly their physiological processes—are somewhat less complicated. Thus, it has been found possible to differentiate between some of the steps in the chain of reactions which lead to cell elongation, so that now it becomes possible to determine the exact structure required in a substance for the regulation of each of these steps.

In consideration of these points experiments have already been carried out by several investigators (1-3). Our own program of research has been under way for some time with a twofold object;

on the one hand, the plant is used as a test object for bioassay to study the relation between chemical structure and physiological activity; on the other, the knowledge thus obtained is used in gaining a better understanding of the physiology of growth in plants. Thus a knowledge of the essential chemical structure of the growth substances should contribute to a knowledge of the substrates with which they react.

As was stated above, the growth of shoots of plants is specifically conditioned by certain substances, the auxins. These auxins are defined physiologically as those substances which give curvature (*i.e.*, growth promotion by cell elongation) in the standard *Avena* test (4). This test is carried out by applying the substance, dissolved in agar, to one side of a decapitated coleoptile of *Avena sativa*. The substance enters at the cut surface and moves longitudinally down that side of the coleoptile to which the agar block is applied. The growth of this side is then promoted, giving rise to a curvature, which, within limits, is proportional to the concentration of the active substance. Standard conditions have been defined and must be adhered to.

Recently another test has been described which also depends upon the promotion of growth on one side of the object. It consists of immersing 4 cm. sections of the etiolated stem of *Pisum* seedlings, longitudinally slit down the center, in the test solution. In active solutions the two halves of the stem curve towards one another, and in this case the curvatures are proportional, within limits, to the logarithm of the concentration of the active substance. All physiological details and literature of both these tests have recently been summarized elsewhere (4). In this "pea test," by contrast with the *Avena* test, the substance does not have to move longitudinally in the tissue but enters everywhere from the solution. Both tests, however, depend primarily upon asymmetrical promotion of growth, in the former by asymmetric application, in the latter by asymmetric response.

Any substance active in the *Avena* test is also active in the pea test; however, a substance active in the pea test may lack certain properties, such as sufficient transportability, and hence be incapable of causing normal curvature in the *Avena* test. Thus the two tests differ in specificity, and a substance possessing growth-promoting activity ("primary activity") is not neces-

sarily active on *Avena* (1). For the sake of simplicity, however, we have considered in this paper only the presence or absence of primary growth-promoting activity; i.e., activity in the pea test. The complications of secondary properties, which allow of the causation of curvature on *Avena*, and probably also affect the quantitative relations in the pea test, will be dealt with in a later paper. In this, the first of a projected series of reports, an attempt is therefore made to present the minimum structural requirements for growth activity.

All the new substances tested for primary activity, and new tests<sup>1</sup> for primary activity of substances previously tested by other techniques, are listed in Table I with an identification number in roman numerals. Table I includes a reference to the method of preparation, or, in the case of substances obtained from other laboratories, an acknowledgment of their source together with an approximate estimate of the activity, expressed as per cent of that of 3-indoleacetic acid. All acids were dissolved in water with the addition of an equivalent amount of NaOH and buffered to pH 5 with diphthalate buffer.

### 1. The Nucleus

*Ring*—The active compounds so far known can be classified into a number of groups according to the nucleus of the molecule. The following are examples of active substances in which a ring is present: auxin *a* (II),  $\Delta^1$ -cyclohexeneacetic acid (VII),  $\alpha$ -toluic acid (phenylacetic acid) (IX), 3-indeneacetic acid (1),  $\alpha$ -naphthaleneacetic acid (XXVII), acenaphtheneacetic acid (29), anthraceneacetic acid (XXVIII), fluoreneacetic acid (29), 1-benzofuraneacetic acid (1), 2-benzofuraneacetic acid (XXIX), 3-indoleacetic acid (heteroauxin) (XXXIII), and 2-thionaphtheneacetic acid (30). From the foregoing it is evident that homocyclic and heterocyclic 5- or 6-membered rings may be present in active substances.

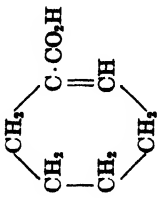
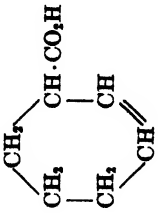
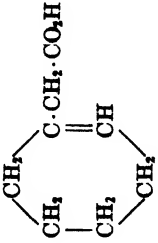
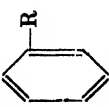
In order to determine whether the presence of a ring system is itself essential for growth activity, it is necessary to know what the other structural requirements are. From the considera-

<sup>1</sup> The biological data were obtained with the assistance of work Project No. 6062, Official Project No. 65-3-5380, conducted under the auspices of the Works Progress Administration.



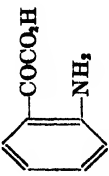
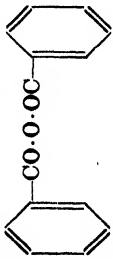
TABLE I  
Summary of New Results, Obtained with Pea Test

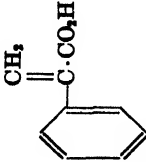
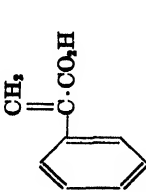
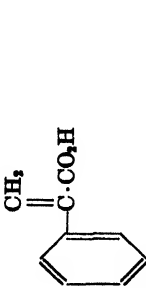
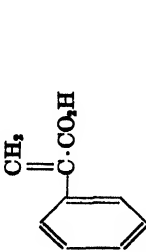
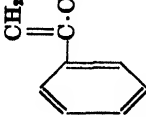
Identification No.	Compound	Source (bibliographic reference No.)	Activity expressed as per cent of that of 3-indoleacetic acid	Remarks
I	$\text{CH}_3\text{:CH}\cdot\text{CH}_2\cdot\text{CO}_2\text{H}$	(5)	Inactive, 0.2 mg. per ml.	For <i>Avena</i> test see (6)
II	$\begin{array}{c} \text{C}_2\text{H}_5 \\   \\ \text{CH}_3\cdot\text{CH}-\text{CH}-\text{C}-\text{CHOH}\cdot\text{CH}_2\cdot(\text{CHOH})_2\text{CO}_2\text{H} \\    \\ \text{CH}_2 \end{array}$ $\begin{array}{c} \text{CH}_3\cdot\text{CH}-\text{CH}-\text{CH} \\    \\ \text{C}_2\text{H}_5 \end{array}$	*	100	
III	$\begin{array}{c} \text{CH}_3 \\ \diagup \quad \diagdown \\ \text{CH}_2 \quad \text{CH}\cdot\text{CH}_2\cdot\text{CO}_2\text{H} \\   \quad   \\ \text{CH}_3 \quad \text{CH}_3 \end{array}$	(7)	Inactive, 0.9 mg. per ml.	
IV	$\begin{array}{c} \text{CH}_3 \\ \diagup \quad \diagdown \\ \text{CH}_2 \quad \text{C:CH}\cdot\text{CO}_2\text{H} \\   \quad   \\ \text{CH}_3 \quad \text{CH}_3 \end{array}$	(7)	" 0.5 " "	

V		(8)	Inactive, 1 mg. per ml.	
VI		(9)	" 1 " "	
VII		(10)	5	<i>Cf.</i> (III)
VIII IX	 R = -CO <sub>2</sub> H " = -CH <sub>2</sub> -CO <sub>2</sub> H		Inactive, 1 mg. per ml. 2	Confirming (3)

\* Gift of Professor Kögl of Utrecht.

TABLE I—Continued

Identification No.	Compound	Source (bibliographic reference No.)	Activity expressed as per cent of that of 8-indoleacetic acid	Remarks
X	R = $-\text{CH}(\text{CH}_3) \cdot \text{CO}_2\text{H}$	(11)	6	Confirming (3)
XI	" = $-\text{C}(\text{CH}_3)_2 \cdot \text{CO}_2\text{H}$	(12)	Inactive, 0.7 mg. per ml.	
XII	" = $-\text{CH}_2 \cdot \text{CH}_2 \cdot \text{CO}_2\text{H}$	(13)	1	
XIII	" = $-\text{CH}_2(\text{CH}_2)_2 \cdot \text{CO}_2\text{H}$	(14)	Inactive, 0.8 mg. per ml.	
XIV	" = $-\text{CH}_2(\text{CH}_2)_3 \cdot \text{CO}_2\text{H}$	†	" 0.5 " " "	
XV	" = $-\text{CHOH} \cdot \text{CO}_2\text{H}$	†	" 0.5 " " "	Also <i>d</i> and <i>l</i> form
XVI	" = $-\text{C}(\text{CH}_3)\text{OH} \cdot \text{CO}_2\text{H}$		" 0.5 " " "	" " " "
XVII	" = $-\text{CH}(\text{CH}_2 \cdot \text{OH}) \cdot \text{CO}_2\text{H}$		"	Reported weakly active (3)
XVIII	" = $-\text{CH}_2\text{COCH}_3$		" 0.9 " " "	<i>dl</i> form
XIX	" = $-\text{CH}_2\text{CONH}_2$		" up to saturated solution	<i>Cf.</i> (IX)
XX		(15)	"	" (IX)
XXI			2	Lower activity than reported (3)
			Inactive up to saturated solution	Reported active on <i>Avena</i> (16)

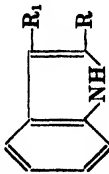
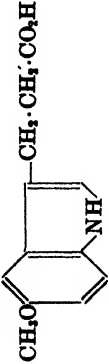
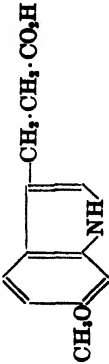
XXII		(17)	2		
XXIII		(18)	10	Inactive, 0.7 mg. per ml.	Lower activity than reported (3); cf. (XXIII)
XXIV		(19)	10	Inactive, 0.3 mg. per ml.	Confirming (3)
XXV		(20)	10		Cf. (XXV)
XXVI		†	100		
XXVII					

† Gift of Professor Alexander McKenzie, University College, Dundee.

‡ Gift of P. W. Zimmerman, Boyce Thompson Institute.

TABLE I—Continued

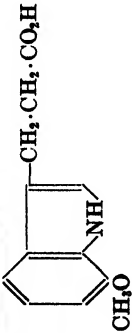
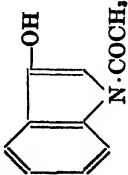
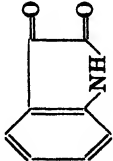
Identification No.	Compound	Source (bibliographic reference No.)	Activity expressed as per cent of that of <i>L</i> -indoleacetic acid	Remarks
XXVIII		†	100	
XXIX		§	15	
XXX				
XXXI	R = -CH <sub>3</sub>	(21)	Inactive, 0.2 mg. per ml.	Reported active on <i>Avena</i> (22)
XXXII	" = -OH	(23)	"	<i>Cf.</i> Section 2
XXXIII	" = -CO <sub>2</sub> H	(24)	"	Confirming (3)
XXXIV	" = -CH <sub>2</sub> -CO <sub>2</sub> H	(25)	100	Standard
XXXV	" = -CH(CH <sub>3</sub> )-CO <sub>2</sub> H	(2)	100	
XXXVI	" = -CH <sub>2</sub> -CH <sub>2</sub> -CO <sub>2</sub> H	(26)	100	Confirming (4)
XXXVII	" = -CH <sub>2</sub> (CH <sub>2</sub> ) <sub>2</sub> -CO <sub>2</sub> H		100	"
			50	(4)

XXXVIII	R = $-\text{CH}\cdot\text{CO}_2\text{H}$   $\text{CH}_2\cdot\text{CO}_2\text{H}$		Inactive, 0.2 mg. per ml.
			
XXXIX	R = $-\text{CO}_2\text{H}$	(27)	Inactive, 0.2 mg. per ml.
XL	R <sub>1</sub> = $-\text{H}$		" 0.2 " "
XL I	R = $-\text{CO}_2\text{H}$		" 0.2 " "
XLII	R <sub>1</sub> = $-\text{CH}_2(\text{CH}_2)_2\cdot\text{CO}_2\text{H}$	(28)	"
	R = $-\text{CO}_2\text{H}$		
	R <sub>1</sub> = $-\text{OH}$		
XLIII			" 0.2 mg. per ml. Cf. (XXXV)
XLIV			" 0.2 " " (XXXV)

§ Gift of Professor T. Reichstein of Zürich.

|| Gift of Dr. R. H. F. Manske, National Research Council, Ottawa.

TABLE I—Concluded

Identification No.	Compound	Source (bibliographic reference No.)	Activity expressed as per cent of that of 3-indoleacetic acid	Remarks
XLV			Inactive, 0.2 mg. per ml.	<i>Cf.</i> (XXXV)
XLVI		(23)	“ 0.1 “ “ “	
XLVII			“ up to saturated solution	<i>Cf.</i> (XX)

tions advanced below it is clear that 3-butenic acid (I) would possess all the characteristics known to be needed for activity with the exception of a ring. This substance was therefore prepared and found to be completely inactive. This, together with the fact that up to now not a single aliphatic acid has been shown to have true growth activity (see the list of compounds found inactive in (3)), strongly indicates that a ring structure is essential.

**Double Bond**—With saturation of the double bond of auxin *a* (II) and auxin *b* (6) or of the  $\Delta^{2,3}$  double bond of 3-indoleacetic acid (XXXIII) (2), all activity on *Avena* disappears. In general only substances possessing an unsaturated ring system have activity. A test of this requirement is given by the series of comparable substances, cyclohexaneacetic acid (III), cyclohexylideneacetic acid (IV), and  $\Delta^1$ -cyclohexeneacetic acid (VII), of which the last mentioned substance, which has the double bond in the ring, alone possesses activity. This strikingly parallels the inactivity of dihydroauxin *a* and pseudoauxin *a*, and the activity of auxin *a*, as described by Kögl and coworkers (31).

**Substituents**—In the present investigation, the effect of nuclear substituents other than the side chain bearing a carboxyl group has not been sufficiently studied to warrant any new conclusions. However, it may be pointed out that the introduction of a methyl group into the 1-, 2-, or 5-position of 3-indoleacetic acid (XXXIII) did not render the resulting compounds inactive (3), while the replacement of the methyl group by an ethyl group in the 2-position of 3-indoleacetic acid gave the inactive 2-ethyl-3-indoleacetic acid (3). Likewise the introduction of the methoxyl group into active 3-indolepropionic acid (XXXV) gave the inactive 5-, 6-, and 7-methoxy-3-indolepropionic acids (XLIII, XLIV, XLV). In the benzene series the amino group has without doubt an important effect. Thus phenylglyoxylic acid (3) is inactive but *o*-aminophenylglyoxylic acid (isatinic acid) (XX) is active. An amino-substituted  $\alpha$ -toluic acid, namely *p*-amino- $\alpha$ -toluic acid, has been reported as active in the pea test (3).

## 2. The Side Chain

**Carboxyl Group**—Up to the present almost all of the active substances known are either carboxylic acids or their esters. The



exceptions noted are tryptamine (32),  $\alpha$ -naphthaleneacetonitrile (29), and auxin *a* lactone (6). Satisfactory evidence for the conversion of tryptamine by the *Avena* coleoptile into an active acid, based essentially upon the fact that its action is delayed, has been given by Skoog (32). Unpublished data indicate that tryptophane behaves in the same way. Similar evidence for the conversion of  $\alpha$ -naphthaleneacetonitrile has been mentioned by Zimmerman and Wilcoxon (29), though on different plant material, and the activity on *Avena* of auxin *a* lactone may safely be ascribed to its hydrolysis in the plant.

In view of the inactivity of indole, the reported activity on *Avena* of skatole (XXX) by Glover (22) seemed questionable, particularly since skatole may be prepared by the decarboxylation of 3-indoleacetic acid. Skatole was therefore prepared by the Fischer synthesis to avoid the possibility of active contaminants, and found to be completely inactive. It would thus seem that the activity of Glover's specimen of skatole must be ascribed to an impurity.

Other indole derivatives lacking a carboxyl group, namely indoxyl (XXXI), 1-acetylindoxyl (XLVI), and isatin (XLVII) are also inactive. That the inactivity of indoxyl was not due to its failure to enter was shown by the observation that after application, either in agar or in solution, the plant showed a blue precipitate of indigo within the cells over a length of about 1 mm. In this connection it may further be noted that benzyl methyl ketone (XVIII), which carries a methyl group in place of the hydroxyl group of the active  $\alpha$ -toluic acid, does not have the slightest activity.

The activity of esters is of considerable interest. Kögl and coworkers showed that the methyl ester of auxin *a* (II) is inactive on *Avena* (6); the esters of 3-indoleacetic acid (XXXIII), on the other hand, are active on *Avena*. This activity has been ascribed by Kögl and Kostermans (2) to the ready hydrolysis of the esters by plant lipases. It is certainly significant that there is no known instance of an inactive carboxylic acid whose ester possesses growth activity (2, 3, 33).

*Position of Carboxyl Group*—There are no special limitations which may be placed on the length of the side chain which carries the carboxyl group. This is evidenced in the indole series by

the activity of 3-indoleacetic acid (XXXIII),  $\alpha$ -methyl-3-indoleacetic acid (XXXIV), 3-indolepropionic acid (XXXV), 3-indolebutyric acid (XXXVI), and 3-indolevaleric acid (XXXVII). In the benzene series only  $\alpha$ -toluic acid (IX), hydratropic acid (X), and hydrocinnamic acid (XII) are active; the higher homologues,  $\gamma$ -phenylbutyric (XIII) and  $\delta$ -phenylvaleric acid (XIV), were inactive. However, the carboxyl group must be removed at least 1 carbon atom from the ring, because the following substances are inactive:  $\Delta^1$ -cyclohexenecarboxylic acid (V),  $\Delta^2$ -cyclohexenecarboxylic acid (VI), benzoic acid (VIII), 2-indolecarboxylic acid (XXXIX), 3-indolecarboxylic acid (XXXII) (25), and indoxyl acid (3-hydroxy-2-indolecarboxylic acid) (XLII). An exception to the above rule was the reported activity of dibenzoyl oxide and dibenzoyl peroxide (XXI) on *Avena* by Snow (16). The latter substance when tested in these laboratories, however, was completely inactive in the pea test and caused a growth inhibition of *Avena*, a phenomenon unrelated to the growth activity under consideration here.

Although the presence of a carboxyl group (or a group readily hydrolyzable to a carboxyl group by the plant) is necessary for growth activity, the presence of more than one appears, in certain instances, to abolish activity. Examples of inactive dicarboxylic acids are 2-carboxy-3-indolepropionic acid (XL), 3-indolesuccinic acid (XXXVIII), and 2-carboxy-3-indolebutyric acid (XLI). The dicarboxylic acids or esters reported active thus far are *m*-phenylenediacetic ethyl ester (3), and indylene-1,3-diacetic acid (34).

**Double Bond**—The following two compounds which have a double bond in the side chain as well as an unsaturated ring are active, *cis*-cinnamic acid (XXIV) (3) and atropic acid (XXII). The corresponding analogues with a reduced side chain, namely hydrocinnamic acid (phenylpropionic acid) (XII) (3) and hydratropic acid (X), are likewise active. Thus the degree of unsaturation of the side chain does not appear to be important, at any rate for qualitative activity.

**Substituents**—The available experimental evidence does not warrant any conclusions with regard to the effect of substituents in the side chain, but the experimental data thus far obtained will be reported at this time. Examples of methyl substitution

in the acetic acid side chain of the benzene series are the active hydratropic acid (X), and the inactive  $\alpha,\alpha$ -dimethyl- $\alpha$ -toluic acid (XI). In the indole series  $\alpha$ -methyl-3-indoleacetic acid (XXXIV) is active but unfortunately attempts to prepare  $\alpha,\alpha$ -dimethyl-3-indoleacetic acid have thus far been unsuccessful.

In view of the fact that the active auxins *a* (II) and *b* have four and two hydroxyl groups respectively in the side chain carrying the carboxyl group, the inactivity of *d*- and *l*-mandelic acid (XV), *d*- and *l*-atrolactic acid (XVI), and *dl*-tropic acid (XVII) is quite remarkable. This fact is of especial interest in that the replacement of the hydroxyl group in each of the inactive compounds mentioned above with hydrogen results in the active  $\alpha$ -toluic acid (IX) in the first instance and the active hydratropic acid (X) in the case of the atrolactic and tropic acids.

### Space Configuration

The importance of *cis-trans* isomerism is clearly indicated by the following relationships.

Active	Inactive
<i>cis</i> -Cinnamic acid (XXIV) (3)	<i>trans</i> -Cinnamic acid (XXIII) (3)
<i>cis-p</i> -Methylcinnamic acid (XXVI)	<i>trans-p</i> -Methylcinnamic acid (XXV)
<i>cis-o</i> -Methoxycinnamic acid (3)	<i>trans-o</i> -Methoxycinnamic acid (3)

The *trans* modifications of *o*-, *m*-, and *p*-nitrocinnamic acid were tested and found to be inactive in the pea test.

### DISCUSSION

The activity of the plant hormones in causing growth may be considered in terms of their chemical structure as it affects the physical properties of the molecule and as it affects the chemical reactivity of the molecule. The direct proportionality between auxin applied and growth induced is good *a priori* evidence that a stoichiometric relation exists and confirmation of this view has been obtained from the pea test described above. Examination of the lowest active concentrations of 3-indoleacetic acid, 3-indolepropionic acid, 3-indolebutyric acid, and  $\alpha$ -naphthaleneacetic acid and auxin *a* has shown that mole for mole their activity is almost the same (4). This strongly indicates that these substances combine directly with some substance in the cell. These facts directed the course of this investigation toward

a correlation of physiological activity with chemical structure rather than with the physical properties of growth-promoting substances. As stated before, the problem has been simplified by not considering the quantitative relationship or degree of activity, which to some extent may perhaps be due to secondary properties (including physical properties).

If the problem is thus simplified and the question limited to one of whether a substance possesses or does not possess the ability to cause cell elongation in plants, then the minimum structural requirements for activity as indicated by the experimental evidence so far reported are (a) a ring system as nucleus, (b) a double bond in this ring, (c) a side chain, (d) a carboxyl group (or a structure readily converted to a carboxyl, such as an ester or nitrile) on this side chain at least 1 carbon atom removed from the ring, (e) a particular space relationship between the ring and the carboxyl group.

The question of space relationship appears to be one of the most important in view of the clear cut evidence offered by the *cis*- and *trans*-cinnamic acids (XXIV, XXIII). The most obvious difference between the *cis*- and *trans*-isomers is the distance between the carboxyl group and the nucleus and this suggests that the growth activity of the *cis*-isomers is occasioned by the close proximity of the carboxyl group to the nucleus. Thus in the  $\alpha$ -toluic acid series of active compounds steric hindrance produced by the introduction of two methyl groups on the  $\alpha$ -carbon atom might account for the inactivity of  $\alpha,\alpha$ -dimethyl- $\alpha$ -toluic acid (XI).

The importance of the space relationship is perhaps supported further by the inactivity of the dicarboxylic acids, 2-carboxy-3-indolepropionic acid (XL) and 2-carboxy-3-indolebutyric acid (XLI) (cf. Section 2). Here also steric hindrance could well prevent the requisite space relationship between the carboxyl group of the side chain and the nucleus in these compounds. Likewise, the inactivity of 2-ethyl-3-indoleacetic acid in contrast to the activity of 2-methyl-3-indoleacetic acid might be ascribed to greater steric hindrance exerted by the ethyl group in the 2-position than by a methyl group in the same position.

In studying the characteristics common to all active substances, we have considered the possibility that an active hydrogen atom

must be present in the side chain bearing the carboxyl group. This would give an obvious explanation for the inactivity of the ring-substituted carboxylic acids such as benzoic and the indole-carboxylic acids, as well as for the striking inactivity of  $\alpha,\alpha$ -dimethyl- $\alpha$ -toluic acid as contrasted with the activity of hydrotropic acid. However, the undoubted activity of isatinic acid (XX) is in conflict with this hypothesis. It is possible that active hydrogen atoms are in fact present in the side chain of isatinic acid, either by hydration of the ketone group, or, more probably, by exchange of hydrogen atoms with those in the ring, involving formation of a quinonoid structure. Some such interchange appears to be indicated by the fact that the presence of the *o*-amino group is necessary for activity to be shown, for phenylglyoxylic acid is inactive. For the present, however, this explanation must be left open.

The activity of apparently wholly unrelated substances such as auxin *a* (II), *cis*-cinnamic acid (XXIV), and 3-indoleacetic acid (XXXIII) has given the impression that no structural specificity exists. From the above analysis it would seem that this view has resulted from the fact that some of the structural requirements of the molecule for growth-promoting activity are comparatively simple, but at the same time it appears that they must be strictly adhered to whether present in a large complex molecule or in a smaller and simpler one. Thus by adhering to the usual chemical classification of organic substances based, for example, on the nucleus, structural specificity in physiological reactions may be lost sight of, although in reality activity evidently depends upon a *combination* of structural characteristics. A striking example of this thesis is afforded by the apparent lack of chemical relationship in the estrogenic substances (35). A parallel may be drawn in another respect between estrogenic and plant growth substances. In both cases there are instances of highly active substances of comparatively simple structure, whereas the naturally occurring estrone and auxin *a* have much more elaborate molecules. Thus the complexity of the molecule is not necessarily connected with its primary activity. In the case of the auxins evidence will be given in later reports that the complexity of the molecule is connected with its secondary properties.

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#### SUMMARY

The specificity of the growth- (cell elongation) promoting properties of a number of synthetic substances have been determined by the use of simple biological tests and evidence is presented that the specificity of physiological activity does not necessarily depend upon the nucleus of a substance but upon a particular molecular configuration. The minimum structural requirements for cell elongation activity in higher plants as indicated by the experimental evidence reported are (a) a ring system as nucleus, (b) a double bond in this ring, (c) a side chain, (d) a carboxyl group (or a structure readily converted to a carboxyl) on this side chain at least 1 carbon atom removed from the ring, and (e) a particular space relationship between the ring and the carboxyl group.

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## THE NUTRITIONAL VALUE OF VARIOUS PROTEIN FRACTIONS OF THE PEANUT ✓

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It has been shown by several workers that the proteins of the whole peanut are adequate for growth (1). On the other hand, Sure (2) showed that the protein, arachin, which makes up over half the total protein of the peanut is inadequate. He was unable to determine the specific amino acids which were deficient, although he supplemented with cystine, tryptophane, alanine, valine, proline, and leucine in various combinations. Some growth was obtained when zein was used as supplement, and excellent growth resulted when lactalbumin was used as supplement to arachin. Conarachin, on the other hand, did not supplement arachin diets.

Jones and Waterman (3) studied the digestibility of arachin *in vitro* and found it slightly less digestible than casein. Cooking improved the digestibility only a little. They suggested that indigestibility might explain the failure of arachin to promote growth, but later work by Mitchell, Burroughs, and Beadles (4) and others did not confirm this indigestibility of peanut proteins *in vivo*.

The nature of the deficiency of arachin was, therefore, not determined. Analyses by Johns and Jones (5) did not disclose any deficiencies, but rather indicated that arachin and especially conarachin, the other globulin of the peanut, contained a surprisingly high proportion of the basic amino acids and adequate amounts of the essential amino acids then known.

In 1932 (6) the writer was fortunate to receive a sample of arachin from Dr. Jones and analyzed it for cystine and methionine. The results showed considerable cystine (1.33 per cent) but only a



very small amount of methionine (0.54 per cent). At this time nothing was known concerning the requirements of the animal for methionine, but Rose *et al.* (7) have recently shown that methionine must be supplied in the food or the animal will not grow.

It was then quite apparent that arachin would probably need a methionine supplement before growth would be possible. The results to be reported establish this.

During the progress of this work Beach and White (8) made a preliminary announcement that they had succeeded in showing methionine to be the limiting nutritive factor of arachin. While our diets differed in several details from theirs, we have confirmed their findings in all respects. Their average daily growth rate of 1.7 gm. is still much below normal growth, which we find to be 3.4 gm. for rats on our basal casein diet. We find that if *l*-tryptophane is added to the methionine-supplemented arachin diet, the average daily growth is increased from 1.75 gm. to 2.3 gm. We have not been able thus far to raise this rate of growth. It may be that threonine or one of the other essential amino acids will have to be added to methionine and tryptophane before normal growth can be obtained on arachin diets.

Sure stated that conarachin did not supplement arachin when used, presumably, in the proportions in which it occurs in the peanut. This we have not confirmed. No experiments have yet been reported in which conarachin has been fed alone. In our experiments we have found conarachin to be an excellent protein for growth. When fed with arachin in the proportions in which it occurs in the extract of the peanut, it supports growth as well as does casein.

In addition to these studies, we have fed the total salt-soluble protein and the whole ground defatted peanut and find that growth rates are nearly the same as those with casein.

#### EXPERIMENTAL

*Preparation of Arachin and Conarachin*—These proteins were prepared from blanched Virginia peanuts according to the directions given by Johns and Jones (9). The average yields of five preparations were arachin 19 per cent and conarachin 5.2 per cent of the meal taken.

Total protein was prepared by extracting the meal as for

arachin, but after filtration of the extract it was dialyzed for 2 days and then evaporated to dryness on a water bath, ground to pass a 40 mesh sieve, and further dried in a vacuum desiccator over  $P_2O_5$ . The yield was 30.0 per cent of the meal taken, which is 84.5 per cent of the protein calculated from the total nitrogen of the meal.

Total globulin was prepared as follows: After the meal was extracted with 10 per cent NaCl, centrifuged, and filtered, the filtrate was dialyzed as usual. The protein which precipitated was filtered off, dried in a current of warm air, and ground (20 mesh). The yield was 21.4 per cent of the meal.

*Diets*—Our diets were made up as follows: protein 20, dextrin 30, sucrose 15, salt mixture (10) 4, agar 2, cod liver oil 5, corn oil 10, milk vitamin concentrate 12, rice polish concentrate 2. Corn oil was substituted for lard, and the amount reduced to improve the consistency and stability of the diet. 19 per cent lard, which we used in our preliminary work, gave a very greasy mixture which turned rancid and unpalatable in a very short time.

We have confirmed Dyer and du Vigneaud (11) with respect to the inadequacy of 12 per cent milk vitamin concentrate, but we prefer to remedy the deficiency by the addition of 2 per cent rice polish concentrate instead of increasing the amount of milk vitamin concentrate.

In making up the diet containing whole peanut we found 56.5 gm. of peanut meal, on calculation from total nitrogen, to contain 20 gm. of protein. This necessitated reducing the dextrin of the basal ration to 3.5 and the sucrose to 5 per cent. The remainder of the diet was the same as the basal.

### *Feeding Experiments*

*Casein Controls*—Six rats given our basal diet containing 20 per cent casein made the usual excellent growth rate which amounted to 3.4 gm. per animal per day.

*Whole Peanut*—Our results with whole, ground, defatted peanut as the source of protein confirm the results of others. The average daily gain was 4.2 gm.

*Total Protein, Total Globulin, and Conarachin*—Owing to the small quantities of these proteins available, only one rat was used for each (Fig. 1). The growth rate on total protein was slightly less than that on whole peanut, but was identical to that on casein.

While no definite conclusions should be drawn from such meager data, it might be profitable to search in the non-extracted nitrogen of whole peanut for essential amino acids. About 85 per cent of the calculated protein of the meal was recovered from the extract.

Total globulin proved to be as good as total protein or casein. This is not in agreement with the statement of Sure. However, differences in variety of peanut and method of preparation, as well as the inadequacy of the data, make accurate comparisons impossible. It appears that our fraction contained enough conarachin with its high methionine and tryptophane contents ade-

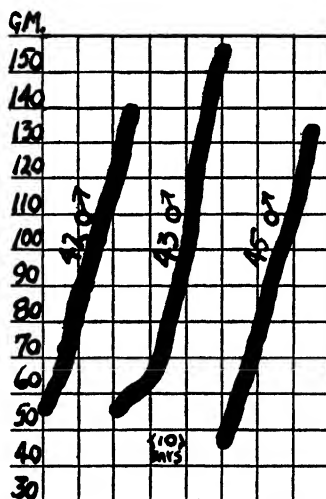


FIG. 1. Diets containing 20 per cent protein. Rat 42 received total protein, Rat 43 total globulin, and Rat 45 conarachin.

quately to supplement arachin. The average yield of several preparations showed a ratio of arachin to conarachin of 4:1.

Conarachin is at least as good as casein at the 20 per cent level. Jones and coworkers (12) find nearly three times as much cystine and tryptophane in conarachin as in arachin. If the non-cystine sulfur of conarachin is methionine sulfur, then conarachin must contain about 1.3 per cent of methionine. This assumption has been shown to be justified as an approximation in over thirty purified proteins (6). No deficiency of basic amino acids was anticipated, since Johns and Jones (13) found conarachin to con-

tain more basic amino acids than any other vegetable protein and nearly as much as muscle itself.

*Arachin*—Nine rats fed the 20 per cent arachin diet showed very poor growth (Fig. 2). Most of them first lost weight for 10 to 20 days, but by the 12th day had regained their original weight. A slow growth then ensued which averaged 0.5 gm. per day. Two males managed to consume sufficient amounts of the ration (Table I) to grow twice as fast as the average, and two others (one female and one male) grew at half the average. The others were quite uniform

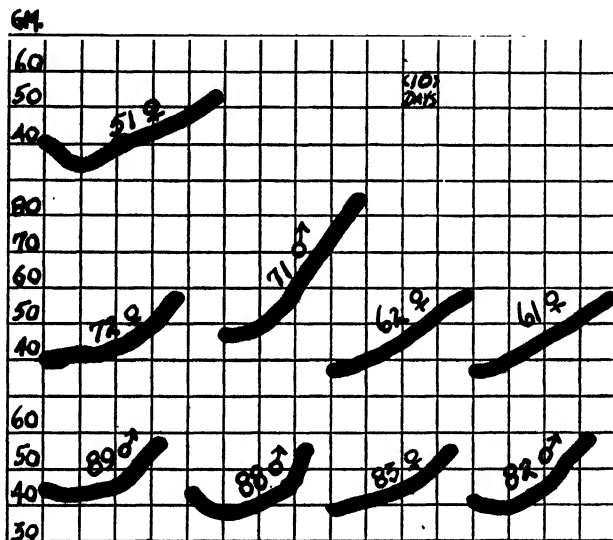


FIG. 2. The inadequacy of arachin

in their growth rates. These results confirm those of all previous workers in showing the inadequacy of this fraction of the peanut.

*Arachin Supplemented with dl-Methionine*—The seven rats shown in Fig. 3 were fed the same diet as the previous group except that 0.53 gm. of *dl*-methionine was added to each 100 gm. of ration. Their average gain was 1.75 gm. per day (confirmation of Beach and White), which, though a striking increase over the unsupplemented diet (0.5 gm. per day), is still only one-half the rate of the casein-fed rats (3.4 gm. per day). This discrepancy between the growth rates on arachin and on casein must therefore be due to a secondary deficiency.

TABLE I  
Average Daily Food Consumption

Rat No.	Sex	Diet	Food consumption <i>gm. per day</i>
41	F.	Whole peanut	10.0
42	M.	Total protein	11.7
43	"	" globulin	10.7
45	"	Conarachin	11.2
89	"	Arachin	5.5
88	"	"	5.1
83	F.	"	6.2
82	M.	"	5.4
72	F.	"	8.1
71	M.	"	7.5
62	F.	"	4.8
61	"	"	9.6
51	"	"	5.8
44	M.	"	7.2
		" + methionine	10.0
841	"	" + "	7.5
		" + " + tryptophane	10.5
84	F.	" + "	9.4
74	M.	" + "	8.2
		" + " + tryptophane	12.6
73	F.	" + "	8.3
64	"	" + "	8.0
		" + " + tryptophane	11.1
63	"	" + "	8.0
56	M.	" + "	7.2
57	"	" + lysine	3.8
		" + methionine + tryptophane	7.6
55	F.	" + tryptophane	2.7
		" + " + methionine	8.0
54	M.	" + lysine	3.3
		" + methionine + tryptophane	9.3
87	F.	" + cystine	5.4
86	M.	" + "	5.5
76	"	" + "	6.4
75	"	" + "	7.4
66	"	" + "	4.8
65	F.	" + "	4.4
53	M.	" + "	5.4

Jones et al. (12) found only 0.88 per cent of tryptophane in arachin, which is a relatively small amount. (Casein has over 2.0 per cent.) When *l*-tryptophane was added to the methionine-supplemented arachin diet, an unmistakable increase in growth rate resulted (Fig. 3, Rats 841, 74, and 64). There is apparently sufficient tryptophane present to support slow growth in the absence of extra methionine, but not enough for rapid growth induced by the methionine supplement (see Fig. 4). It is to be noted that Sure found supplements of cystine and tryptophane were ineffec-

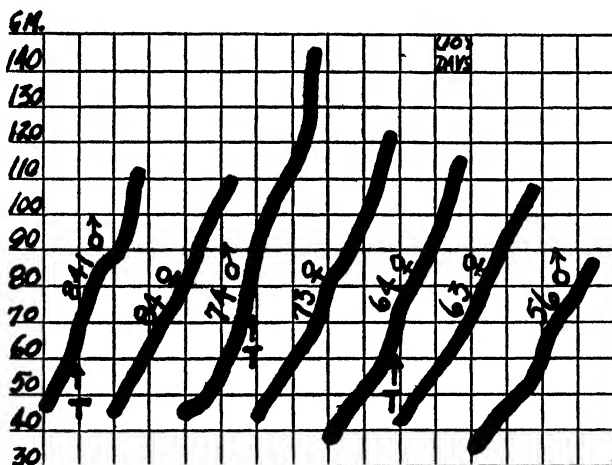


FIG. 3. Arachin supplemented with methionine and tryptophane. 0.53 gm. of *dl*-methionine was added to each 100 gm. of ration. This amount raises the methionine content of the diet to that of the casein diet. Rats 841, 74, and 64 received in addition 0.26 gm. of *l*-tryptophane per 100 gm. of ration at *T*. This amount raises the tryptophane content of the ration to that of the casein diet.

tive in stimulating growth, showing the truly secondary character of the tryptophane deficiency of arachin. This we have confirmed.

In order to clarify this problem further, Rat 55 (Fig. 4) was fed arachin plus tryptophane from the beginning. No increase in weight occurred until methionine was added on the 25th day.

Because there was some doubt (5) as to the actual amount of lysine in arachin (4.98 per cent by the Van Slyke method and 1.7 per cent by the Kossel and Kutscher method) we fed two rats

arachin plus *d*-lysine hydrochloride for 30 days. No growth resulted until the animals were changed to the methionine-tryptophane diet. Unfortunately, we neglected to continue the rats on lysine for

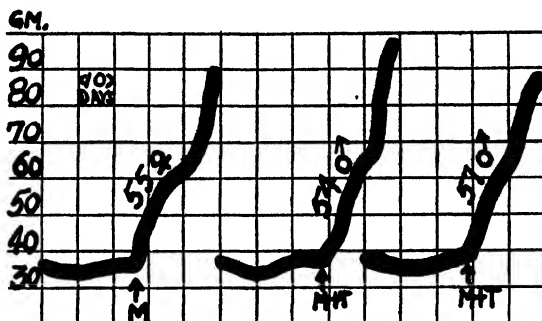


FIG. 4. Arachin diet supplemented as shown below. Rat 55 received 0.26 gm. of *l*-tryptophane per 100 gm. of ration. At *M* 0.53 gm. of *dl*-methionine was added to each 100 gm. of ration. Rats 54 and 57 received 1.47 gm. of *d*-lysine hydrochloride per 100 gm. of ration. At *M + T* 0.53 gm. of *dl*-methionine and 0.26 gm. of *l*-tryptophane were added to each 100 gm. of ration and the lysine was omitted.

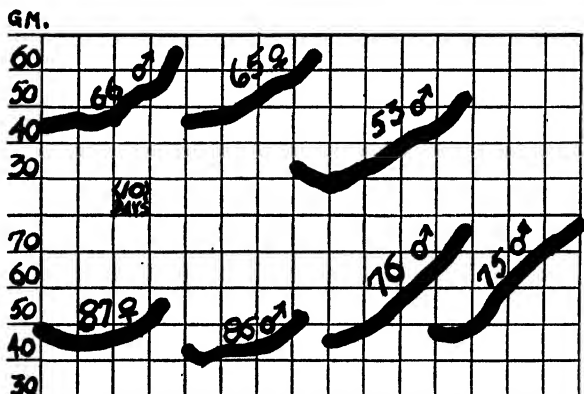


FIG. 5. Arachin supplemented with *l*-cystine. 0.43 gm. of *l*-cystine was added to each 100 gm. of ration. This amount of cystine carries sulfur equivalent to the methionine added to the diets of the rats shown in Fig. 5.

then we might have improved the growth rate still more and shown whether or not lysine was the third deficiency.

The arachin diet differs from the low casein diet in that the

latter can be made adequate by either cystine or methionine supplements, whereas the former can be improved only by methionine. Fig. 5 shows the growth curves of seven rats fed arachin plus cystine. These are practically identical to the curves in Fig. 2, showing the rates on unsupplemented arachin. This confirms the findings of Sure and of Beach and White.

#### SUMMARY

These studies show that whole, defatted peanut, total protein, and total globulin are approximately equivalent to casein in promoting growth of the white rat when fed at a 20 per cent level.

Conarachin is an excellent protein for growth when fed as the only source of protein, and when fed with arachin as total globulin is still as effective as is casein.

Arachin, which makes up four-fifths of the total globulin, is very poor. The addition of methionine greatly improves the arachin ration, and the further addition of tryptophane produces still better growth.

Neither tryptophane, lysine, nor cystine improves arachin diets in the absence of methionine supplement.

The fact that the growth rate on the casein diet is faster than that on the supplemented arachin diet indicates a third deficiency in arachin.

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## INDEX TO AUTHORS

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### A

- Andrews, James C.** Reduction of certain sulfur compounds to hydrogen sulfide by the intestinal microorganisms of the dog, 687
- Antaki, Albert.** See KERR and ANTAKI, 49
- Avrin, Ira.** See BERNSTEIN, JONES, ERICKSON, WILLIAMS, AVRIN, and MACY, 507
- See ERICKSON, WILLIAMS, BERNSTEIN, AVRIN, JONES, and MACY, 515

### B

- Babbitt, Dorothea.** See MARSHALL, 263
- Baernstein, Harry D.** The nutritional value of various protein fractions of the peanut, 781
- Balls, A. K., Matlack, M. B., and Tucker, I. W.** The hydrolysis of glycerides by crude pancreas lipase, 125
- Beach, Elliot F.** See WHITE and BEACH, 219
- Bergmann, Max.** Complex salts of amino acids and peptides. III. Salts of dioxalatodipyridinochromiato acid (dioxpyridic acid) and dioxalatodianilinochromiato acid (dioxanilic acid), 569

- Bergmann, Max, and Niemann, Carl.** On the structure of silk fibroin, 577
- Bernstein, Samuel S., Jones, Robert L., Erickson, Betty Nims, Williams, Harold H., Avrin, Ira, and Macy, Icie G.** A method for the preparation of posthemolytic residue or stroma of erythrocytes, 507
- See ERICKSON, WILLIAMS, BERNSTEIN, AVRIN, JONES, and MACY, 515
- Bibler, Walter G.** See EICHELBERGER, 323
- Block, Richard J.** See JACKSON and BLOCK, 425
- Boyd, Eldon M., and Stevenson, J. W.** The regeneration of blood lipids following a single massive hemorrhage in rabbits, 147
- Boyden, Ruth, and Potter, V. R.** On the form of copper in blood plasma, 285
- Brown, W. L.** The influence of pimiento pigments on the color of the egg yolk of fowls, 655
- Butler, Allan M.** See MINDLIN and BUTLER, 673

### C

- Campbell, Harold A., and Link, Karl Paul.** Acetals in the

- sugar group. I. The dimethyl acetal of *d*-galactose, 635
- Carpenter, Thorne M. The partition of urinary nitrogen of fasting and hibernating woodchucks (*Arctomys monax*), 343
- Carter, Herbert E. See WEST and CARTER, 611
- See WEST, KRUMMEL, and CARTER, 605
- Chaikoff, I. L. See LORENZ, ENTENMAN, and CHAIKOFF, 619
- See PERLMAN, RUBEN, and CHAIKOFF, 169
- Chargaff, Erwin, and Olson, Kenneth B. Studies on the chemistry of blood coagulation. VI. Studies on the action of heparin and other anticoagulants. The influence of protamine on the anticoagulant effect *in vivo*, 153
- Christman, Clarence C. See LEVENE and CHRISTMAN, 203, 661
- Corey, Edward L. See EHRENSTEIN and COREY, 297
- Corley, Ralph C. See SNYDER and CORLEY, 491
- Craig, Lyman C. See JACOBS and CRAIG, 419

## D

- Doty, J. R., and Eaton, A. G. Metabolism of the basic amino acids. I. Rates of absorption in rats of the monohydrochlorides of *d*-lysine and *L*-histidine, 139

## E

- Eaton, A. G. See DOTY and EATON, 139
- Ehrenstein, Maximilian, and Corey, Edward L. Activation of testosterone by higher fatty acids and their acid sodium salts, 297
- Eichelberger, Lillian. The exchange of salt and water between muscle and blood. IV. Correction of values for volume phases of skeletal muscle. Methods for determination of blood volume in muscle, 323
- Elvehjem, C. A. See SCHANTZ, ELVEHJEM, and HART, 381
- Emerson, Gladys A. See EMERSON, EMERSON, MOHAMMAD, and EVANS, 99
- Emerson, Oliver H., Emerson, Gladys A., Mohammad, Ali, and Evans, Herbert M. The chemistry of vitamin E. Tocopherols from various sources, 99
- Entenman, C. See LORENZ, ENTENMAN, and CHAIKOFF, 619
- Erickson, Betty Nims, Williams, Harold H., Bernstein, Samuel S., Avrin, Ira, Jones, Robert L., and Macy, Icie G. The lipid distribution of posthemolytic residue or stroma of erythrocytes, 515
- See BERNSTEIN, JONES, ERICKSON, WILLIAMS, AVRIN, and MACY, 07
- Evans, Herbert M. See EMERSON, EMERSON, MOHAMMAD, and EVANS, 99

- Evelyn, Kenneth A., and Gibson, John G., 2nd.** A new type of absorption cell for the photoelectric microcolorimeter, 391  
 —. See MALLOY and EVELYN, 597

G

- Gersdorff, Charles E. F.** See JONES, GERSDORFF, and PHILLIPS, 745  
**Gibson, John G., 2nd.** See EVELYN and GIBSON, 391  
**Gildea, Edwin F.** See MAN and GILDEA, 77  
**Goebel, Walther F.** Derivatives of glucuronic acid. VIII. The structure of benzoylglucuronic acid, 649  
**Goerner, Alfred.** Effect of dibenzanthracene on vitamin A and total lipid of mitochondria, 529  
**Graham, W. R., Jr.** The utilization of lactic acid by the lactating mammary gland, 1  
 —, **Peterson, V. E., Houchin, O. B., and Turner, C. W.** The utilization of fractions of the nitrogen partition of the blood by the active mammary gland, 275  
**Greenberg, David M.** See TUFTS and GREENBERG, 693, 715

H

- Harrer, Carter J.** See STOTZ, HARRER, SCHULTZE, and KING, 407  
**Hart, E. B.** See SCHANTZ, ELVEHJEM, and HART, 381

- Hartzler, Eva R.** A note on the determination of potassium by the method of Shohl and Bennett, 19  
**Hess, W. C.** See SULLIVAN and HESS, 11  
**Heymann, Walter.** Metabolism and mode of action of vitamin D. IV. Importance of bile in the absorption and excretion of vitamin D, 249  
 —. V. Intestinal excretion of vitamin D, 257  
**Hilditch, T. P., and Longenecker, H. E.** Further determination and characterization of the component acids of butter fat, 497  
**Hirschmann, H., and Wintersteiner, Oskar.** The isolation of estrogenic diols from the urine of pregnant mares, 303  
**Houchin, O. B.** See GRAHAM, PETERSON, HOUCHIN, and TURNER, 275

I

- Iob, Vivian, and Swanson, W. W.** The extracellular and intracellular water in bone and cartilage, 485  
**Irish, Oliver J.** See DU VIGNEAUD and IRISH, 349

J

- Jackson, Elizabeth B.** See MORISON, MCLEAN, and JACKSON, 439  
**Jackson, Richard W., and Block, Richard J.** The metabolism of cystine and methionine. II. The availability of *d*- and *l*-methionine and their formyl

- derivatives in the promotion of growth, 425
- Jacobs, Walter A., and Craig Lyman C.** The ergot alkaloids. XIII. The precursors of pyruvic and isobutyrylformic acids, 419
- Johnson, Marvin J.** Specificity of intestinal aminopolypeptidase, 89
- Jones, D. Breese, Gersdorff, Charles E. F., and Phillips, Sammie.** Proteins of the black bean of the Mayas, *Phaseolus vulgaris*, 745
- Jones, Robert L.** See BERNSTEIN, JONES, ERICKSON, WILLIAMS, AVRIN, and MACY, 507
- See ERICKSON, WILLIAMS, BERNSTEIN, AVRIN, JONES, and MACY, 515
- K**
- Kerr, Stanley E.** Note on the phosphorus content of rat brain in experimental rickets, 53
- and **Antaki, Albert.** The carbohydrate metabolism of brain. V. The effect of certain narcotics and convulsant drugs upon the carbohydrate and phosphocreatine content of rabbit brain, 49
- Keston, Albert S., Rittenberg, D., and Schoenheimer, Rudolf.** Determination of deuterium in organic compounds, 227
- King, C. G.** See SCHULTZE, STOTZ, and KING, 395
- See STOTZ, HARRER, SCHULTZE, and KING, 407
- Kochakian, Charles D., MacLachlan, P. L., and McEwen, H. Douglas.** Do androgens affect the blood lipids? 433
- Koepfli, J. B., Thimann, Kenneth V., and Went, F. W.** Phytohormones: structure and physiological activity. I, 763
- Kreider, Leonard C.** See LEVENE, TIPSON, and KREIDER, 199
- Krummel, Guy S.** See WEST, KRUMMEL, and CARTER, 605
- Kuna, Martin.** See LEVENE and KUNA, 291
- Kurtz, Alton C.** A simple synthesis of *dl*-citrulline, 477
- L**
- Levene, P. A., and Christman, Clarence C.** Conversion of uronic acids into corresponding hexoses. III. Catalytic reduction and deacetylation of the methyl ester of 2,3,4-triacetyl  $\alpha$ -methyl-*d*-galacturonide, 203
- and —. IV. Catalytic reduction of the methyl ester of diacetone *d*-galacturonic acid, 661
- and **Kuna, Martin.** The configurational relationship of 3-aminoheptane to that of norleucine, 291
- , **Tipson, R. Stuart, and Kreider, Leonard C.** Conversion of uronic acids into corresponding hexoses. II. Catalytic reduction of the methyl ester of 2,3,4-trimethyl  $\alpha$ -methyl-*d*-galacturonide, 199

- Lineweaver, Hans.** The solubility and chemical and physical absorption of nitrogen gas in *Azotobacter* cells, 549
- Link, Karl Paul.** See CAMPBELL and LINK, 635
- Longenecker, H. E.** See HILDITCH and LONGENECKER, 497
- Lorenz, F. W., Entenman, C., and Chaikoff, I. L.** The influence of age, sex, and ovarian activity on the blood lipids of the domestic fowl, 619

M

- MacLachlan, P. L.** See KOCHAKIAN, MACLACHLAN, and MCEWEN, 433
- Macy, Icie G.** See BERNSTEIN, JONES, ERICKSON, WILLIAMS, AVRIN, and MACY, 507
- See ERICKSON, WILLIAMS, BERNSTEIN, AVRIN, JONES, and MACY, 515
- Malloy, Helga Tait, and Evelyn, Kenneth A.** Oxidation method for bilirubin determinations in bile and meconium with the photoelectric colorimeter, 597
- Man, Evelyn B., and Gildea, Edwin F.** Notes on the extraction and saponification of lipids from blood and blood serum, 77
- Marshall, E. K., Jr.** Determination of sulfanilamide in blood and urine, 263
- Matlack, M. B.** See BALLS, MATLACK, and TUCKER, 125
- Mattill, H. A.** See SCHULTZ and MATTILL, 183

- McEwen, H. Douglas.** See KOCHAKIAN, MACLACHLAN, and MCEWEN, 433
- McLean, Regina.** See MORISON, MCLEAN, and JACKSON, 439
- Miller, Max.** The diffusible calcium of serum and transudates *in vivo*, 59
- Ionized calcium of serum and transudates *in vivo*, 71
- Mindlin, Rowland L., and Butler, Allan M.** The determination of ascorbic acid in plasma; a macromethod and micro-method, 673
- Mohammad, Ali.** See EMERSON, EMERSON, MOHAMMAD, and EVANS, 99
- Morison, Robert S., McLean, Regina, and Jackson, Elizabeth B.** Observations on the relation between ionized and total calcium in normal and abnormal sera and their ultrafiltrates, 439
- Moyer, Laurence S.** Electrokinetic aspects of surface chemistry. III. A comparison of the microscopic and moving boundary methods of electrophoresis, 641

N

- Niemann, Carl.** See BERGMANN and NIEMANN, 577

O

- Olson, Kenneth B.** See CHARGAFF and OLSON, 153

P

- Perlman, I., Ruben, S., and Chaikoff, I. L.** Radioactive

- phosphorus as an indicator of phospholipid metabolism. I. The rate of formation and destruction of phospholipids in the fasting rat, 169
- Peterson, V. E. See GRAHAM, PETERSON, HOUCHIN, and TURNER, 275
- Peterson, W. H. See WOOLLEY and PETERSON, 207, 213
- Phillips, Sammie. See JONES, GERSDORFF, and PHILLIPS, 745
- Potter, V. R. See BOYDEN and POTTER, 285
- R**
- Richards, Marianna M. The effect of glycine upon the activity coefficient of glycine, egg albumin, and carboxy-hemoglobin, 727
- Rittenberg, D. See KESTON, RITTENBERG, and SCHOENHEIMER, 227
- Ruben, S. See PERLMAN, RUBEN, and CHAIKOFF, 169
- S**
- Salomon, Kurt. See STERN and SALOMON, 461
- Schantz, E. J., Elvehjem, C. A., and Hart, E. B. The relation of fat to the utilization of lactose in milk, 381
- Schmidt, E. G. The determination of sulfanilamide in tungstic acid blood filtrates by means of sodium  $\beta$ -naphthoquinone-4-sulfonate, 757
- Schoenheimer, Rudolf. See KESTON, RITTENBERG, and SCHOENHEIMER, 227
- Schultz, Harold W., and Mattill, H. A. Studies on the vitamin B complex, 183
- Schultze, M. O., Stotz, Elmer, and King, C. G. Studies on the reduction of dehydroascorbic acid by guinea pig tissues, 395
- See STOTZ, HARRER, SCHULTZE, and KING, 407
- Scudi, John V. The determination of sulfanilamide (*p*-aminobenzenesulfonamide) in biological media, 539
- Sklersky, Samuel. See SOBEL and SKLERSKY, 665
- Smith, Elizabeth R. B. See SMITH, TAYLOR, and SMITH, 109
- Smith, Paul K., Taylor, Alice C., and Smith, Elizabeth R. B. Thermodynamic properties of solutions of amino acids and related substances. III. The ionization of aliphatic amino acids in aqueous solution from one to fifty degrees, 109
- Snyder, Fred H., and Corley, Ralph C. Amino acid catabolism. V. The influence of structural configuration on the deamination of  $\alpha$ -amino acids in the normal dog, 491
- Snyder, J. C. A note on the use of the Haldane apparatus for the analysis of gases containing ether vapor, 21
- Sobel, Albert E., and Sklersky, Samuel. A direct acidimetric microtitration method for calcium, 665

- Stekol, Jakob A.** Studies on the mercapturic acid synthesis in animals. VII. Bromobenzene and *L*-cystine in relation to growth of rats on a navy bean meal diet, 55
- VIII. *L*-Cystine, *dl*-methionine, glutathione, and taurine in relation to the synthesis of mercapturic acids in the rat, 333
- Stern, Kurt G., and Salomon, Kurt.** On ovoverdin, the carotenoid-protein pigment of the egg of the lobster, 461
- and **White, Abraham.** Studies on the constitution of insulin. III. The acetylation of insulin by ketene, 371
- Stevenson, J. W.** See **BOYD and STEVENSON**, 147
- Stotz, Elmer, Harrer, Carter J., Schultze, M. O., and King, C. G.** The oxidation of ascorbic acid in the presence of guinea pig liver, 407
- See **SCHULTZE, STOTZ, and KING**, 395
- Sullivan, M. X., and Hess, W. C.** The effect of pyruvic acid on the estimation of cystine and cysteine, 11
- Swanson, W. W.** See **IOB and SWANSON**, 485
- T**
- Taylor, Alice C.** See **SMITH, TAYLOR, and SMITH**, 109
- Thimann, Kenneth V.** See **KOEPFLI, THIMANN, and WENT**, 763
- Tipson, R. Stuart.** See **LEVENE, TIPSON, and KREIDER**, 199
- Toennies, Gerrit.** Oxidation of cysteine in non-aqueous media. The "sulfenic acid" as the primary oxidation product, 27
- Tucker, I. W.** See **BALLS, MTLACK, and TUCKER**, 125
- Tufts, Elma V., and Greenberg, David M.** The biochemistry of magnesium deficiency. I. Chemical changes resulting from magnesium deprivation, 693
- and — II. The minimum magnesium requirement for growth, gestation, and lactation, and the effect of the dietary calcium level thereon, 715
- Turner, C. W.** See **GRAHAM, PETERSON, HOUCHIN, and TURNER**, 275
- V**
- du Vigneaud, Vincent, and Irish, Oliver J.** The rôle of the acetyl derivative as an intermediary stage in the biological synthesis of amino acids from keto acids, 349
- W**
- Wald, George, and Zussman, Hyman.** Carotenoids of the chicken retina, 449
- Went, F. W.** See **KOEPFLI, THIMANN, and WENT**, 763
- West, Harold D., and Carter, Herbert E.** Synthesis of  $\alpha$ -amino- $\beta$ -hydroxy-*n*-butyric acids. VI. Preparation of *d*-



- and *l*-allothreonine and nutritive value of the four isomers, 611
- West, Harold D., Krummel, Guy S., and Carter, Herbert E.** Synthesis of  $\alpha$ -amino- $\beta$ -hydroxy-*n*-butyric acids. V. Preparation of *dl*-allothreonine, with notes concerning the addition of methyl hypobromite to unsaturated acids, 605
- White, Abraham, and Beach, Eliot F.** The rôle of cystine, methionine, and homocystine in the nutrition of the rat, 219
- See STERN and WHITE, 371
- Williams, Harold H.** See BERNSTEIN, JONES, ERICKSON, WILLIAMS, AVRIN, and MACY, 507
- See ERICKSON, WILLIAMS, BERNSTEIN, AVRIN, JONES, and MACY, 515
- Wintersteiner, Oskar.** See HIRSCHMANN and WINTERSTEINER, 303
- Woolley, D. W., and Peterson, W. H.** The chemistry of mold tissue. XIV. Isolation of cyclic choline sulfate from *Aspergillus sydowi*, 213
- and —. Some observations on the Kapeller-Adler method for the determination of histidine. The histidine content of yeast, 207
- Wyckoff, Ralph W. G.** An ultracentrifugal study of the pH stability of tobacco mosaic virus protein, 239

## Z

- Zussman, Hyman.** See WALD and ZUSSMAN, 449

## INDEX TO SUBJECTS

### A

- Acetals:** Sugars (CAMPBELL and LINK) 635
- Acid(s):** Unsaturated, methyl hypobromite effect (WEST, KRUMMEL, and CARTER) 605
- Age:** Blood lipids, effect (LORENZ, ENTENMAN, and CHAIKOFF) 619
- Albumin:** Egg, activity coefficient, glycine effect (RICHARDS) 727
- Alkaloids:** Ergot (JACOBS and CRAIG) 419
- Allothreonine:** *d*-, preparation and nutrition value (WEST and CARTER) 611
- dl*-, preparation (WEST, KRUMMEL, and CARTER) 605
- l*-, preparation and nutrition value (WEST and CARTER) 611
- Amino acid(s):**  $\alpha$ -, deamination, structural configuration influence (SNYDER and CORLEY) 491
- Aliphatic, ionization, aqueous solution (SMITH, TAYLOR, and SMITH) 109
- Basic, metabolism (DOTY and EATON) 139
- Catabolism (SNYDER and CORLEY) 491

### Amino acid(s)—*continued*:

- Keto acid synthesis, acetyl derivative, rôle (DU VIGNEAUD and IRISH) 349
- Related substances, solutions, properties (SMITH, TAYLOR, and SMITH) 109
- Salts, complex (BERGMANN) 569
- Solutions, thermodynamic properties (SMITH, TAYLOR, and SMITH) 109
- Aminoheptane:** 3-, norleucine, configurational relationship (LEVENE and KUNA) 291
- Amino -  $\beta$  - hydroxy - *n* - butyric acids:**  $\alpha$ -, synthesis (WEST, KRUMMEL, and CARTER) 605
- (WEST and CARTER) 611
- Aminopolypeptidase:** Intestine, specificity (JOHNSON) 89
- Androgens:** Blood lipids, effect (KOCHAKIAN, MACLACHLAN, and McEWEN) 433
- Anticoagulants:** Blood, action (CHARGAFF and OLSON) 153
- Apparatus:** Colorimeter, micro-, photoelectric, absorption cell (EVELYN and GIBSON) 391
- Haldane, ether - containing gases (SNYDER) 21

- Ascorbic acid:** Blood plasma, determination (MINDLIN and BUTLER) 673  
 Dehydro-, reduction, guinea pig tissue (SCHULTZE, STOTZ, and KING) 395  
 Oxidation, liver effect (STOTZ, HARRER, SCHULTZE, and KING) 407
- Aspergillus sydowi:** Choline sulfate, cyclic, isolation (WOOLLEY and PETERSON) 213
- Azotobacter:** Nitrogen gas, solubility and absorption (LINEWEAVER) 549
- B**
- Bean:** Black, proteins (JONES, GERSDORFF, and PHILLIPS) 745  
 Navy, meal, bromobenzene and l-cystine supplement, growth effect (STEKOL) 55
- Benzenesulfonamide:** *p*-Amino-, biological media, determination (SCUDI) 539
- Benzoylglucuronic acid:** Chemical constitution (GOEBEL) 649
- Bile:** Bilirubin determination, oxidative, photoelectric colorimeter (MALLOY and EVELYN) 597  
 Vitamin D absorption and excretion, effect (HEYMANN) 249
- Bilirubin:** Bile, determination, oxidative, photoelectric colorimeter (MALLOY and EVELYN) 597  
 Meconium, determination, oxidative, photoelectric col-
- Bilirubin—continued:**  
 orimeter (MALLOY and EVELYN) 597
- Blood:** Anticoagulant effect, protamine effect (CHARGAFF and OLSON) 153  
 Coagulation, heparin and anticoagulants, action (CHARGAFF and OLSON) 153  
 Lipids, age, sex, and ovarian activity, effect (LORENZ, ENTENMAN, and CHAIKOFF) 619  
 —, androgens, effect (KOCHAKIAN, MACLACHLAN, and MC-EWEN) 433  
 —, extraction and saponification (MAN and GILDEA) 77  
 —, hemorrhage effect (BOYD and STEVENSON) 147  
 Muscle, volume determination (EICHELBERGER) 323  
 Salt and water, muscle and, exchange (EICHELBERGER) 323
- Blood cell:** Red, posthemolytic residue, lipid distribution (ERICKSON, WILLIAMS, BERNSTEIN, AVRIN, JONES, and MACY) 515  
 —, —, preparation (BERNSTEIN, JONES, ERICKSON, WILLIAMS, AVRIN, and MACY) 507
- Blood serum:** Lipids, extraction and saponification (MAN and GILDEA) 77
- Bone:** Water, extracellular and intracellular (IOB and SWANSON) 485
- Brain:** Carbohydrate metabolism (KERR and ANTAKI) 49  
 —, narcotics and convulsant

**Brain—continued:**

- drugs, effect (KERR and ANTAKI) 49  
 Phosphocreatine, narcotics and convulsant drugs, effect (KERR and ANTAKI) 49  
 Phosphorus, rickets (KERR) 53  
 Bromobenzene: Dietary, growth effect (STEKOL) 55  
 Butter fat: Acids, determination and characterization (HILDITCH and LONGENECKER) 497  
 Butyric acids:  $\alpha$ -Amino- $\beta$ -hydroxy-*n*-, synthesis (WEST, KRUMMEL, and CARTER) 605  
 (WEST and CARTER) 611

**C**

- Calcium: Determination, acidimetric microtitration (SOBEL and SKLERSKY) 665  
 Diffusible, blood serum and transudates *in vivo* (MILLER) 59  
 Ionized and total, blood serum and ultrafiltrates, relation (MORISON, MCLEAN, and JACKSON) 439  
 —, blood serum and transudates *in vivo* (MILLER) 71  
 Magnesium requirement, growth, gestation, and lactation, relation (TUFTS and GREENBERG) 715  
 Carbohydrate: Brain, narcotics and convulsant drugs, effect (KERR and ANTAKI) 49  
 Metabolism, brain (KERR and ANTAKI) 49

- Carboxyhemoglobin: Activity coefficient, glycine effect (RICHARDS) 727  
 Carotenoids: Retina, chicken (WALD and ZUSSMAN) 449  
 Cartilage: Water, extracellular and intracellular (IOB and SWANSON) 485  
 Choline sulfate: Cyclic, *Aspergillus sydowi*, isolation (WOOLLEY and PETERSON) 213  
 Citrulline: *dl*-, synthesis (KURTZ) 477  
 Clotting: Blood, anticoagulant effect, protamine effect (CHARGAFF and OLSON) 153  
 —, heparin and anticoagulants, action (CHARGAFF and OLSON) 153  
 Colorimeter: Micro-, photoelectric, absorption cell (EVELYN and GIBSON) 391  
 Copper: Blood plasma (BOYDEN and POTTER) 285  
 Cysteine: Determination, pyruvic acid effect (SULLIVAN and HESS) 11  
 Oxidation, non-aqueous media (TOENNIES) 27  
 Sulfenic acid, oxidation product, non-aqueous media (TOENNIES) 27  
 Cystine: Determination, pyruvic acid effect (SULLIVAN and HESS) 11  
 Homo-, nutrition, rôle (WHITE and BEACH) 219  
*l*-, dietary, growth effect (STEKOL) 55  
 —, mercapturic acid synthesis, relation (STEKOL) 333

**Cystine—continued:**

- Metabolism (JACKSON and BLOCK) 425  
 Nutrition, rôle (WHITE and BEACH) 219

**D**

- Dehydroascorbic acid:** Reduction, guinea pig tissue (SCHULTZE, STOTZ, and KING) 395  
**Deuterium:** Organic compounds, determination (KESTON, RITTENBERG, and SCHOENHEIMER) 227  
**Diacetone *d*-galacturonic acid:** Methyl ester, catalytic reduction (LEVENE and CHRISTMAN) 661  
**Dibenzanthracene:** Lipid, total, mitochondria, effect (GOERNER) 529  
 Vitamin A, mitochondria, effect (GOERNER) 529  
**Dioxalatodianilinochromiato acid:** Salts (BERGMANN) 569  
**Dioxalatodipyridinochromiato acid:** Salts (BERGMANN) 569  
**Drugs:** Convulsant, brain carbohydrate and phosphocreatine, effect (KERR and AN-TAKI) 49

**E**

- : Albumin, activity coefficient, glycine effect (RICHARDS) 727  
 Lobster, ovoverdin (STERN and SALOMON) 461  
 Yolk color, pimiento pigments, effect (BROWN) 655

**Electrokinetics:** Surface chemistry (MOYER) 641

**Electrophoresis:** Measurements, microscopic and moving boundary methods, comparison (MOYER) 641

**Enzyme(s):** *See also* Aminopoly-peptidase, Lipase

**Ergot:** Alkaloids (JACOBS and CRAIG) 419

**Erythrocyte:** *See* Blood cell, red

**Estrogenic diols:** Urine, pregnant mares, isolation (HIRSCHMANN and WINTERSTEINER) 303

**Ether:** -Containing gases, Hal-dane apparatus (SNYDER) 21

**F**

**Fasting:** Phospholipid formation and destruction (PERLMAN, RUBEN, and CHAIKOFF) 169

Urine nitrogen partition (CARPENTER) 343

**Fat:** Milk lactose utilization relation (SCHANTZ, ELVEHJEM, and HART) 381

**Fatty acid(s):** Higher, and acid sodium salts, testosterone activation (EHRENSTEIN and COREY) 297

**Fibroin:** Silk, structure (BERGMANN and NIEMANN) 577

**Formic acid:** Isobutyryl-, precursors (JACOBS and CRAIG) 419

**G**

**Galactose:** *d*-, dimethyl acetal (CAMPBELL and LINK) 635

- Galacturonic acid:** *d*-, diacetone, methyl ester, catalytic reduction (LEVENE and CHRISTMAN) 661
- Galacturonide:** 2,3,4-Triacetyl  $\alpha$ -methyl-*d*-, methyl ester, catalytic reduction and deacetylation (LEVENE and CHRISTMAN) 203
- 2,3,4-Trimethyl  $\alpha$ -methyl-*d*-, methyl ester, catalytic reduction (LEVENE, TIPSON, and KREIDER) 199
- Gas:** Ether-containing, Haldane apparatus (SNYDER) 21
- Gestation:** Magnesium requirement, calcium relation (TUFTS and GREENBERG) 715
- Glucuronic acid:** Benzoyl-, chemical constitution (GOEBEL) 649
- Derivatives (GOEBEL) 649
- Glutathione:** Mercapturic acid synthesis, relation (STEKOL) 333
- Glycerides:** Hydrolysis, pancreas lipase, crude (BALLS, MALLACK, and TUCKER) 125
- Glycine:** Activity coefficient, glycine effect (RICHARDS) 727
- Carboxyhemoglobin activity coefficient, effect (RICHARDS) 727
- Egg albumin activity coefficient, effect (RICHARDS) 727
- Growth:** Bromobenzene, dietary, effect (STEKOL) 55
- l*-Cystine, dietary, effect (STEKOL) 55
- Growth—continued:**
- Magnesium requirement, calcium relation (TUFTS and GREENBERG) 715
- d*-Methionine and formyl derivatives, effect (JACKSON and BLOCK) 425
- l*-Methionine and formyl derivatives, effect (JACKSON and BLOCK) 425
- H**
- Haldane:** Apparatus, ether-containing gases (SNYDER) 21
- Hemoglobin:** Carboxy-, activity coefficient, glycine effect (RICHARDS) 727
- Heparin:** Blood anticoagulant action (CHARGAFF and OLSON) 153
- Heptane:** 3-Amino-, norleucine, configurational relationship (LEVENE and KUNA) 291
- Hexoses:** Uronic acid conversion (LEVENE, TIPSON, and KREIDER) 199
- (LEVENE and CHRISTMAN) 203, 661
- Hibernation:** Urine nitrogen partition (CARPENTER) 343
- Histidine:** *d*-, monohydrochloride, absorption rate (DOTY and EATON) 139
- Yeast, determination. Kappeller-Adler method (WOOLLEY and PETERSON) 207
- Homocystine:** Nutrition, rôle (WHITE and BEACH) 219
- Hormone:** Phyto-, structure and physiological activity (KOPFLI, THIMANN, and WENT) 763

**Hydrogen ion concentration:** Tobacco mosaic virus protein, stability (WYCKOFF) 239

**Hydrogen sulfide:** Sulfur compounds reduced to, intestinal microorganism (ANDREWS) 687

## I

**Insulin:** Acetylation, ketene (STERN and WHITE) 371

Chemical constitution (STERN and WHITE) 371

**Intestine:** Aminopolypeptidase specificity (JOHNSON) 89

Microorganism, sulfur compounds reduced to hydrogen sulfide (ANDREWS) 687

Vitamin D excretion (HEY-MANN) 257

**Isobutyrylformic acid:** Precursors (JACOBS and CRAIG) 419

## K

**Ketene:** Insulin acetylation (STERN and WHITE) 371

**Keto acids:** Amino acid synthesis from, acetyl derivative, rôle (DU VIGNEAUD and IRISH) 349

## L

**Lactation:** Lactic acid utilization, mammary gland (GRAHAM) 1

Magnesium requirement, calcium relation (TUFTS and GREENBERG) 715

**Lactic acid:** Mammary gland, lactating, utilization (GRAHAM) 1

**Lactose:** Milk, utilization, fat relation (SCHANTZ, ELVEHJEM, and HART) 381

**Leucine:** Nor-, 3-aminoheptane, configurational relationship (LEVENE and KUNA) 291

**Lipase:** Pancreas, crude, glyceride hydrolysis (BALLS, MATLACK, and TUCKER) 125

**Lipid(s):** Blood, age, sex, and ovarian activity, effect (LORENZ, ENTENMAN, and CHAIKOFF) 619

—, androgens, effect (KOCHAKIAN, MACLACHLAN, and McEWEN) 433

— cell, red, posthemolytic residue, distribution (ERICKSON, WILLIAMS, BERNSTEIN, AVRIN, JONES, and MACY) 515

—, extraction and saponification (MAN and GILDEA) 77

—, hemorrhage effect (BOYD and STEVENSON) 147

— serum, extraction and saponification (MAN and GILDEA) 77

Total, mitochondria, dibenzanthracene effect (GOERNER) 529

**Liver:** Ascorbic acid oxidation, effect (STOTZ, HARRER, SCHULTZE, and KING) 407

**Lobster:** Egg, ovoverdin (STERN and SALOMON) 461

**Lysine:** d-, monohydrochloride, absorption rate (DOTY and EATON) 139

## M

- Magnesium:** Deficiency (TUFTS and GREENBERG) 693, 715  
 Gestation requirement, calcium relation (TUFTS and GREENBERG) 715  
 Growth requirement, calcium relation (TUFTS and GREENBERG) 715  
 Lactation requirement, calcium relation (TUFTS and GREENBERG) 715  
**Mammary gland:** Blood nitrogen partition, utilization (GRAHAM, PETERSON, HOUGHIN, and TURNER) 275  
 Lactating, lactic acid utilization (GRAHAM) 1  
**Meconium:** Bilirubin determination, oxidative, photoelectric colorimeter (MALLOY and EVELYN) 597  
**Mercapturic acid:** Synthesis, animals (STEKOL) 55  
 —, *l*-cystine, *dl*-methionine, glutathione, and taurine, relation (STEKOL) 333  
**Metabolism:** Carbohydrate, brain (KERR and ANTAKI) 49  
 Phospholipid, radioactive phosphorus as indicator (PERLMAN, RUBEN, and CHAIKOFF) 169  
**Methionine:** *d*-, and formyl derivatives, growth effect (JACKSON and BLOCK) 425  
*dl*-, mercapturic acid synthesis, relation (STEKOL) 333  
*l*-, and formyl derivatives, growth effect (JACKSON and BLOCK) 425

## Methionine

## M:

- Metabolism (JACKSON and BLOCK) 425  
 Nutrition, rôle (WHITE and BEACH) 219  
**Methyl hypobromite:** Unsaturated acids, effect (WEST, KRUMMEL, and CARTER) 605  
**Microorganism:** Intestine, sulfur compounds reduced to hydrogen sulfide by (ANDREWS) 687  
**Mitochondria:** Lipid, total, dibenzanthracene effect (GOERNER) 529  
 Vitamin A, dibenzanthracene effect (GOERNER) 529  
**Mold:** Tissue, chemistry (WOOLLEY and PETERSON) 213  
*See also Aspergillus sydowii*  
**Mosaic:** Tobacco, virus protein, hydrogen ion concentration stability (WYCKOFF) 239  
**Muscle:** Blood volume determination (EICHELBERGER) 323  
 Salt and water, blood and, exchange (EICHELBERGER) 323  
 Skeletal, salt and water exchange, volume phases, correction (EICHELBERGER) 323

## N

- Narcotics:** Brain carbohydrate and phosphocreatine, effect (KERR and ANTAKI) 49  
**Nitrogen:** Gas, *Azotobacter*, solubility and absorption (LINEWEAVER) 549  
 Partition, blood, mammary



**Nitrogen—continued:**

gland, utilization (GRAHAM,  
PETERSON, HOUCHIN, and  
TURNER) 275

Urine, partition, fasting and  
hibernation, effect (CAR-  
PENTER) 343

**Norleucine:** 3 - Aminoheptane,  
configurational relationship  
(LEVENE and KUNA) 291

**O**

**Organic compounds:** Deuterium,  
determination (KESTON, RIT-  
TENBERG, and SCHOEN-  
HEIMER) 227

**Ovary:** Activity, blood lipids,  
effect (LORENZ, ENTENMAN,  
and CHAIKOFF) 619

**Ovooverdin:** Lobster egg (STERN  
and SALOMON) 461

**P**

**Pancreas:** Lipase, crude, glyc-  
eride hydrolysis (BALLS,  
MATLACK, and TUCKER)  
125

**Peanut:** Proteins, nutritive value  
(BAERNSTEIN) 781

**Peptidase:** Aminopoly-, intes-  
tine, specificity (JOHNSON)  
89

**Peptides:** Salts, complex (BERG-  
MANN) 569

**Phaseolus vulgaris:** *See* Bean,  
black

**Phosphocreatine:** Brain, narcot-  
ics and convulsant drugs,  
effect (KERR and ANTAKI)  
49

**Phospholipid(s):** Formation and  
destruction, fasting animal

**Phospholipid(s)—continued:**

(PERLMAN, RUBEN, and  
CHAIKOFF) 169

Metabolism, radioactive phos-  
phorus as indicator (PERL-  
MAN, RUBEN, and CHAI-  
KOFF) 169

**Phosphorus:** Brain, rickets  
(KERR) 53

Radioactive, phospholipid me-  
tabolism indicator (PERL-  
MAN, RUBEN, and CHAI-  
KOFF) 169

**Pigment:** Carotenoid-protein,  
lobster egg (STERN and  
SALOMON) 461

Pimiento, egg yolk color, effect  
(BROWN) 655

**Pimiento:** Pigments, egg yolk  
color, effect (BROWN) 655

**Plant:** Hormones, structure and  
physiological activity (KOEP-  
FLI, THIMANN, and WENT)  
763

**Potassium:** Determination,  
Shohl-Bennett method  
(HARTZLER) 19

**Pregnancy:** Urine, estrogenic  
diols, isolation (HIRSCH-  
MANN and WINTERSTEINER)  
303

*See also* Gestation

**Protamine:** Blood anticoagulants,  
effect (CHARGAFF and  
OLSON) 153

**Protein(s):** Bean, black (JONES,  
GERSDORFF, and PHILLIPS)  
745

Peanut, nutritive value  
(BAERNSTEIN) 781

Tobacco, mosaic virus, hy-  
drogen ion concentration sta-  
bility (WYCKOFF) 239

- Pyruvic acid:** Cysteine determination, effect (SULLIVAN and HESS) 11  
 Cystine determination, effect (SULLIVAN and HESS) 11  
 Precursors (JACOBS and CRAIG) 419

## R

- Retina:** Carotenoids, chicken (WALD and ZUSSMAN) 449  
**Rickets:** Brain phosphorus (KERR) 53

## S

- Salt:** Blood and muscle, exchange (EICHELBERGER) 323  
**Sex:** Blood lipids, effect (LORENZ, ENTENMAN, and CHAIKOFF) 619  
**Silk:** Fibroin, structure (BERGMANN and NIEMANN) 577  
**Sugar(s):** Acetals (CAMPBELL and LINK) 635  
**Sulfanilamide:** Biological media, determination (SCUDI) 539  
     Blood, determination (MARSHALL) 263  
     — filtrates, determination, sodium  $\beta$ -naphthoquinone-4-sulfonate (SCHMIDT) 757  
     Urine, determination (MARSHALL) 263  
**Sulfenic acid:** Cysteine oxidation product, non-aqueous media (TOENNIES) 27  
**Sulfur:** Compounds, reduction to hydrogen sulfide, intestinal microorganism (ANDREWS) 687

## T

- Taurine:** Mercapturic acid synthesis, relation (STEKOL) 333  
**Testosterone:** Activation, fatty acids, higher, and acid sodium salts (EHRENSTEIN and COREY) 297  
**Threonine:** *d*-Allo-, preparation and nutrition value (WEST and CARTER) 611  
     *dl*-Allo-, preparation (WEST, KRUMMEL, and CARTER) 605  
     *l*-Allo-, preparation and nutrition value (WEST and CARTER) 611  
**Tobacco:** Mosaic virus protein, hydrogen ion concentration stability (WYCKOFF) 239  
**Tocopherols:** Chemistry (EMERSON, EMERSON, MOHAMMAD, and EVANS) 99  
**Transudate:** Calcium, diffusible, *in vivo* (MILLER) 59  
     —, ionized, *in vivo* (MILLER) 71  
**2,3,4-Triacetyl  $\alpha$ -methyl-*d*-galacturonide:** Methyl ester, catalytic reduction and deacetylation (LEVENE and CHRISTMAN) 203  
**2,3,4-Trimethyl  $\alpha$ -methyl-*d*-galacturonide:** Methyl ester, catalytic reduction (LEVENE, TIPSON, and KREIDER) 199

## U

- Urine:** Estrogenic diols, pregnant mares, isolation (HIRSCHMANN and WINTERSTEINER) 303

**Uronic acids:** Hexoses, conversion (LEVENE, TIPSON, and KREIDER) 199  
(LEVENE and CHRISTMAN) 203, 661

## V

**Virus:** Tobacco mosaic protein, hydrogen ion concentration stability (WYCKOFF) 239  
**Vitamin:** A, mitochondria, dibenzanthracene effect (GOERNER) 529  
B complex (SCHULTZ and MATTILL) 183  
D absorption and excretion, bile effect (HEYMANN) 249  
—, excretion, intestine (HEYMANN) 257

**Vitamin—continued:**

E, chemistry (EMERSON, EMERSON, MOHAMMAD, and EVANS) 99

## W

**Water:** Blood and muscle, exchange (EICHELBERGER) 323  
Extracellular and intracellular, bone and cartilage (IOB and SWANSON) 485

## Y

**Yeast:** Histidine determination, Kapeller-Adler method (WOOLLEY and PETERSON) 207





